

Time to champion global data

Environmental threats makes the need for enhanced atmospheric monitoring obvious, but the will and organization to fund it are insufficient. Less obviously, global monitoring of fundamental geophysical parameters would bring great benefits.

MOST busy people have on their desks problems that somehow never reach the top of the pile. The time required to deal with them might be short, yet somehow there is always something more urgent (or —let's be honest — more interesting) to get on with.

In *Nature's* offices, of course, this rare phenomenon tends to be restricted to internal memoranda. More interestingly, and more worryingly, something rather similar is a persistent problem in the geosciences. Nobody can quite get round to meeting one fundamental need: adequately and consistently recording, year on year, streams of data that may be individually prosaic but which cumulatively provide many of the best indicators of how our planet is constructed and is behaving. Geophysics and geochemistry (in their broadest senses, encompassing the solid Earth, the hydrosphere and the atmosphere) are much the worse for this chronic failure.

Occasionally a community gathers itself for a monumental effort. A notable early example was the International Geophysical Year (IGY), conceived by James van Allen and others in 1950, and implemented in 1957–58 with the participation of 67 countries. As it happened, during the IGY the first satellites were launched and nuclear weapons were exploded at high altitudes: related civil and military research both led to considerable new understanding of the Earth's magnetic and outer atmospheric environment. But the sheer global reach of observation was also a significant achievement. The International Geosphere–Biosphere Programme and the World Ocean Circulation Experiment are two of its successors, both established with quite specific research goals of global scope — respectively investigating geochemical cycles and fluxes between ocean basins.

There are other phenomena that need to be monitored in a less goal-directed spirit but no less purposefully. Take atmospheric aerosols. Their main sources at the surface and in the lower atmosphere are known, as are their important roles in radiative transfer (and hence climate) and in chemistry (and hence pollution). But details of those roles are inadequately understood, as are their temporal and spatial behaviour. As Meinrat Andreae, an atmospheric chemist, emphasized in last week's issue (*Nature* 380, 389–390; 1996), because of a lack of systematic measurements we cannot quantify the contribution from human activities over past decades — a significant lacuna in atmospheric modelling, and one that is likely to persist.

Again, the problem is a world-wide one. The US National Oceanic and Atmospheric Administration maintains a few monitoring stations, and the World Meteorological Organisation (WMO) is also making a significant contribution. There are field projects to examine particular aspects of aerosol behaviour — for example, coordinated measurements by satellite, airborne and ground-based experiments are being conducted this summer on the US East Coast to examine the radiative effects of sulphate aerosols. But the lower-profile long-term monitoring surely deserves greater support than it is getting.

The significant variability of aerosol distributions means that relatively dense monitoring networks are desirable. But other scientifically important phenomena suffer from overdense observations in some parts of the globe and a paucity elsewhere. This problem has been highlighted in a paper from the Geophysical Research Centre in Potsdam, Germany (I. I. Mueller *et al.*, submit-

ted to the *Journal of Geodynamics*). It usefully summarizes the conceptual links between a variety of geophysical parameters that each require global monitoring: gravity, surface and internal deformations of the Earth, dynamic interactions between the Earth, oceans and atmosphere, the planet's rotation, not to mention magnetic fields. Techniques being applied include satellite laser ranging, very-long-baseline interferometry, the Global Positioning System, seismology, tide and ground-water gauges, and superconducting gravimeters.

A glance at a global map of the distribution of such monitoring activities reveals unsurprising but problematic variations: concentrations in Europe and the United States and voids in less developed countries and in the vast expanse of the Pacific Ocean. Yet, as the authors emphasize, optimizing the distributions of the various networks would enhance by an order of magnitude the measurement precision of tectonic plate velocities and gravity parameters, for example. It would also open to experiment, among several processes, the relationship between ocean topography, ocean dynamics and the gravitational potential, as well as motions in the Earth's core detectable by variations of its rotation vector.

The organization that looks after such challenges is the International Union of Geodesy and Geophysics (IUGG). It has recently begun to look sympathetically at the issue, but there are obstacles. Possessiveness may be one. Which of the eight institutions in Western Europe with superconducting gravimeters is going to be sufficiently internationally minded to have its gravimeter sent off to somewhere in the Southern Hemisphere — which in its entirety hosts just one? And who would pay for maintenance and data processing at the new sites? That is a question of funding capacity but also of principle — should one country, comparatively well resourced but hard pressed, support a scientific facility based in another? Only if the science is sufficiently high up the list of the donor's national priorities — which seems questionable when it comes to a pricy monitoring station that forms but one element of a multinational network. And there will also be a tab to be picked up by the recipients.

Regrettably, the need to build (or redistribute) global networks comes at a time when the budgetary environment is rapidly worsening. Two years ago, for example, the US National Aeronautics and Space Administration (NASA) was operating a set of six laser-ranging stations at a cost of \$6 million a year. Now that budget has collapsed, and NASA is seeking to dispose of the equipment to anybody who might usefully take it on, also offering just one year of technical support — Tahiti is one possible new location.

Lacking the immediate environmental relevance of aerosols, and potentially involving substantial international transfers of technological resources that amount to a form of foreign aid, the task of filling those observational gaps presents the geophysical community with something of a political challenge, to put it mildly. Without the recent backing of the IUGG, there would have been no hope of progress. A modest target is that, three years down the road, a well-distributed network of 10 multi-instrumented geophysical reference stations might be in operation. The scientific benefits are clear, but whether some coalition of agencies can muster sufficient resolve to achieve even that development remains questionable. That is a sorry state of affairs. □



German science body complains of excessive regulation of research

Munich. The over-regulation of science in Germany is undermining the efficiency with which research is carried out, and could threaten the country's scientific competitiveness, according to a report from the Deutsche Forschungsgemeinschaft (DFG) — the German Science Council — which is due to be published next month.

An interdisciplinary group of experts appointed by the DFG to look at legal restrictions on researchers points out that laws governing activities such as embryo research, data protection and the use of laboratory animals and genetic technologies are much stricter in Germany than in other countries.

In addition, it says that civil servants enforcing the laws can be excessively zealous, often unfairly denying or delaying the approval of scientific experiments. In 1993, for example, local authorities responsible for licensing genetic engineering experiments twice ignored the recommendations of the Central Committee for Biological Safety, which evaluates experimental protocols, and required extra safety precautions, leading to additional restrictions and paper work.

The report also points out that government officials similarly overrode scientific advisers when, in 1992, they denied a licence to researchers at the University of Kiel who had received a contract from the federal office of forestry and agriculture to find more efficient ways to exterminate brown rats during periods of infestation. The officials involved had argued that plans to catch and tag 40 rats, in order to monitor their movements in the wild, would inflict unjustifiable stress on the rats.

Scientists argue that such cases of official zeal undermine the 'freedom of research' that is guaranteed in Germany's post-Second-World-War constitution. Two years ago, the federal constitutional court ruled in a test case that licensing authorities did not have the competence to assess the scientific aims of a research project. Nevertheless, the DFG report says that federal and regional officials continue to impose their moral judgements on projects.

Many feel that this tendency increases the problem of long delays in licensing procedures. Germany's genetic engineering regulations were recently changed to speed up the average time of licence approval. In theory, this should reduce the average time for approval from ten weeks to six. But in practice, says the report, this period can be extended by officials who have the right to request further information at any point during the application procedure.

Such licensing delays cause particular difficulties for biologists using animals in

experiments. According to a survey carried out by Peter Gruss, a molecular geneticist at the Max Planck Institute for Biophysical Chemistry in Göttingen, scientists have to wait an average of five months for a licence for such experiments. "This means that, as experimentalists, we cannot react to a scientific report for nearly half a year," he says. "That is detrimental to our competitiveness."

The report points out a further problem, that laws designed primarily for industry — such as those governing emissions and chemical usage and disposal — also apply to

research, making the approval process for new facilities, for example, both complicated and lengthy.

The DFG report suggests that such laws should be modified to match the actual conditions under which research facilities operate. It points out, for example, that while industrial companies tend to produce large amounts of a relatively small number of chemicals, a research laboratory tends to have small amounts of a large number of chemicals. Yet both are treated equally by the authorities when it comes to applying for an operating licence. **Alison Abbott**

Hydrogen research 'facing a vacuum'

Washington. The recent rapid expansion in US support for research into the use of hydrogen as a fuel could come to a sudden end when Robert Walker (Republican, Pennsylvania), chair of the Science Committee in the House of Representatives, retires



Walker: has backed long crusade

in November, the annual meeting of the National Hydrogen Association was told last week.

Spending on hydrogen research at the Department of Energy (DOE) has shot up from just over \$1 million in 1993 to \$14 million this year, largely as a result of Walker's relentless support. In the same period, more substantial programmes for research into other renewable energy sources have been sharply curtailed: support for solar energy, for example, fell this year from \$259 million to \$192 million.

Alan Lloyd of the Desert Research Institute, Reno, Nevada, the next chairman of the DOE's hydrogen advisory board, told last week's meeting that unless supporters of hydrogen research organize themselves much more effectively, "there's going to be a vacuum" when Walker leaves.

The Clinton administration does not see hydrogen as a priority, and has requested that its budget be cut back to \$11 million next year. "There is a major gap between the Department of Energy's public statements on hydrogen, and its budget requests to Congress," says Lloyd. Neil Rossmessl, hydrogen programme manager at the DOE, concedes that the department is "providing the minimum amount of funding needed to keep this technology moving".

The House has passed a bill, sponsored by Walker, that would authorize \$100 mil-

lion for hydrogen research over three years. A companion measure in the Senate has bipartisan sponsorship from Jon Kyl (Republican, Arizona) and Tom Harkin (Democrat, Iowa). There is a reasonable prospect that the Senate, which rarely bothers with research authorization bills, will pass the bill in recognition of Walker's long crusade for hydrogen. But even that would not guarantee future funding.

Hydrogen advocates want research at every stage of a complex energy chain, from electric production of hydrogen through its storage, transportation and distribution, as well as on fuel cells and engines, and the vehicles they would power. Such challenges are mostly technical rather than scientific. But Walker calls them "basic research", arguing that "we are not picking a technology, we are picking an area of study" which

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

End of a dream? hydrogen's potential suffered a setback when the Hindenburg exploded in 1937

has "been dramatically under-emphasized by the Department of Energy".

Kazukiyo Okano, director of research at Japan's World Energy Network programme, told the meeting that Japan will spend \$18 million this year on hydrogen research: it is undertaking an accelerating, \$100-million research programme in the period 1993-98, which may lead on to a \$3-billion technology development and demonstration programme. **Colin Macilwain**

China pledges 12 per cent science boost

London. China has promised to increase its spending on research and development by 12 per cent in the current (1996) financial year, two percentage points more than the planned overall increase in government spending. It has also renewed a pledge to treble spending on science and technology as a proportion of gross domestic product (GDP) by the end of the century.

In a speech last month to the People's Congress, Liu Zhongli, the finance minister, said "greater efforts must be made beginning this year" to reduce expenditure and to pursue a "tight financial policy" aimed at addressing the country's budget deficit.

But he emphasized that the budget for science and technology — currently 0.5 per

cent of GDP — would continue to increase. In particular, spending on science and education, will grow by 12 per cent to 10.7 billion yuan (US\$1.28 billion) and 99.4 billion yuan respectively. Both these increases are 2 per cent higher than the overall increase in government spending, which is planned to be 1.2 per cent lower than the increase in revenue. China's budget deficit, which last year climbed by 31 per cent, is projected to increase by a further 26 per cent this year.

Liu acknowledged that the increases in science expenditure came at a time when many departments had "already exerted themselves" to reduce spending. But he emphasized that the extra funds for science, technology and education would

be spent more efficiently. "More spending for education and science and technology", he said, "should be used to improve and upgrade existing education and research facilities, instead of increasing projects or new institutes".

In 1994, China spent a total of 22.2 billion yuan on research and development, approximately one quarter of which came from the private sector. The government is aiming to increase industry's share of research and development to at least 50 per cent by 2000 (see *Nature* 378, 542; 1995). In 1992, China spent 0.7 per cent of GDP on research and development. The country's rapid economic growth has contributed to the decline in this figure. Ehsan Masood

Prospects of power prompt rethink for German Greens

Munich. As the German Green party — arguably the most powerful environmentalist force in Europe — seeks to establish its credibility as a potential coalition partner in a future federal government, it remains split over its opposition to biotechnology and genetic engineering, just as it is over attempts to move closer to the centre-stage of German politics.

Those seeking more moderate policies include Manuel Kiper, spokesman for the party's parliamentary group on research policy. Kiper argues that a blanket ban on biotechnology is inappropriate, both because, he claims, its many benefits — particularly in health and environment — outweigh any environmental threats, and because biotechnology products are already being marketed with the approval of grassroots environmental groups.

But Kiper's position, which he estimates to have significant support in the party, continues to encounter stiff resistance from the fundamentalist wing, which is arguing against any change of policy. Marina Steindorff, the parliamentary group's spokeswoman on health, energetically argues that the party should insist on the withdrawal of all biotechnological products from the market, and accuses Kiper of political opportunism.

Kiper, a molecular biologist by training, says that he is particularly concerned that 'fundamentalism' in the party has been blocking for more than a year a planned party conference on the use of genetically engineered enzymes in washing powder. During this period, other environmental and consumer organizations have reached agreement with the washing powder industry on safety issues and labelling.

Indeed, the parliamentary group appears to agree with Kiper that the party should at least review its position, and a debate on this issue is now likely to take place at the annual

conference in spring next year. The party has already agreed, at its 1996 annual conference last month, to lift a ten-year boycott of computers. The boycott was so out of touch with society that it raised smiles rather than serious support, says Kiper, arguing that the ban on biotechnology will be seen as similarly ridiculous in future.

Another critic is Jens Reich, a molecular biologist at the Max Delbrück Centre for Molecular Medicine in Berlin, who, although not himself a member of the party, was picked as its candidate for the presidential elections two years ago. Reich argues that the fundamentalists' uncompromising



Kiper: argues against ban on biotechnology.

attitude to biotechnology has cost the party votes. But he claims that their influence is on the wane. "There is a growing readiness to accept that ecological goals can be reached without having to get rid of all technology," he says.

Both of the main political parties, the ruling Christian Democrats and the opposition Social Democrats, are keen to reverse popular opposition to biotechnology and genetic engineering as part of their efforts to boost economic growth. Scientists notice that pressure groups are now much more prepared to discuss controversial issues rather than dismiss them dogmatically.

At a meeting held last month by Dachema, a company that promotes the interests of Germany's chemical industries, politicians from both the Christian Democrat and Social Democrat parties showed

themselves quick to exploit the more tolerant environment. In particular, there was widespread support for the view that attempts to establish a consensus — Germany's traditional political approach — on biotechnology should be abandoned. "In a pluralistic society it is acceptable to live with a bit of dissent," said Wolf-Michael Catenhusen, the Social Democrats' spokesman on research.

Meanwhile, the Greens are preparing themselves for possible power-sharing in a future federal government. They already share government with the Social Democrats in three of Germany's 16 Länder, and after last week's regional elections are likely to join them in a fourth coalition, in Schleswig-Holstein. Conflicts within these coalitions about biotechnology have so far been avoided by an unspoken agreement not to raise the issue; internal arguments have tended to focus on more immediate issues such as transport.

There have long been talks at the federal level between the Social Democrats and the Greens about a possible coalition after the 1998 federal elections. Now the Social Democrats might have a competitor. Two weeks ago, sensitive to the uncertain fortunes of the Free Democrats, the Christian Democrats' current coalition partner, despite their reasonable success in the recent regional elections, Chancellor Helmut Kohl stated that he would also "not rule out" a future coalition with the Greens.

Joschka Fischer, head of the Greens' parliamentary group, is actively trying to make his party attractive to both suitors. But he is aware that, in order to do so, the party will have to shed its extremist image, and that includes abandoning a blanket ban on genetic engineering which, according to recent opinion polls, no longer has public sympathy. Alison Abbott

Engineering academy moves to oust president

Washington. The US National Academy of Engineering (NAE) is trying to oust from office Harold Liebowitz, its recently elected president, in a move that opens the way for a public — and potentially damaging — battle for the soul of the organization.

At a meeting on 29 March, the 20-member council of the academy passed a vote of no confidence in Liebowitz as the organization's president, and submitted to the members an amendment to the NAE's by-laws which, if passed, would enable them to vote Liebowitz out of office.

Liebowitz immediately promised to fight the proposed amendment. In a strongly worded ten-page statement, he accused the council of having stifled his work as soon as

he was elected last July, and promised to rescue the NAE from alleged domination by the National Academy of Sciences (NAS), its larger and older sister organization.

Both academies share the same operating body, the National Research Council. But the NAS has ultimate responsibility for the entire complex. Last month, the NRC stripped Liebowitz from his position as its vice-chair, opening the way for his removal from the NAE (see *Nature* 379, 761; 1996).

"I do not accept that the NAE should be subservient to the National Academy of Sciences," Liebowitz says in his statement. He claims that the two academies had "failed effectively to oppose cuts in engineering" and that science "had sold out engineering

in recent budget debates".

Liebowitz, who was elected by a narrow margin as NAE president on a 'write-in' vote last year, after being excluded from the ballot, also argues that other officers of the academy "have been unwilling, from the outset, to work with me because I am an outsider".

But Morris Tanenbaum, vice president of the NAE and a former senior manager at AT&T, says that he and other officials have done everything possible to cooperate with Liebowitz. "I don't know anything more that we could have done," he says. "My conscience is clear."

Tanenbaum concedes that NAE's operations have been "negatively affected" by the Liebowitz row. Bill Colglazier, executive officer of the NRC, says that the flow of study requests to the academy complex has not yet slowed down as a result. But "there is certainly a potential worry that this might damage the credibility of the academies", he says.

Staff and officials of the academy complex attribute the conflict to Liebowitz's personal behaviour. But the embattled president prefers to blame it on the political relationship between the NAE and the NAS.

Indeed, the subservience of engineering to science — real or imagined — is an emotive issue for the 1,800 senior professors and industrialists who make up the NAE's elite membership. But having flirted with rebellion last July, it seems inconceivable that they will back Liebowitz this time round.

NAE members are due to receive their ballot forms on the proposed amendments to the by-laws this week. If these are passed, a resolution to remove Liebowitz could be voted on as early as next month.

Colin Macilwain

\$60 million for genome sequencing

Washington. The US National Institutes of Health (NIH) announced this week the long-anticipated launch of a \$60-million pilot project to explore the technical and economic feasibility of large-scale genome sequencing technology. If successful, this could lead to the sequencing of the entire human genome by the year 2005.

Six research groups are to share a total of \$18 million in the current financial year, which ends in September, to investigate a range of sequencing and related technologies. In line with proposals first put forward last year (see *Nature* 375, 93; 1995) these will be aiming for an accuracy of one error in 10,000 bases.

"The pilot project will help us determine whether it will be necessary to strive for 99.99 per cent accuracy when we scale up, and whether it can be done cost-effectively with the sequencing strategies emerging during the next three years," says Mark Guyer, assistant director of the National Center for Human Genome Research (NCHGR).

The centre says that it is "encouraging" those receiving sequencing grants to release preliminary DNA sequence information "within a few days or weeks of its discovery". This is longer than a preliminary, controversial release time of about one day, endorsed at an informal meeting of sequencing team leaders held in Bermuda last month, but criticized as being insufficient to study the potential intellectual property implications (see *Nature* 380, 279; 1996).

Nevertheless, the NCHGR points out that this is considerably faster than research information is usually released. "The tremendous value of this data for disease research justifies this aggressive policy," says the centre, adding that the "finished" sequence, with all data "double-and-triple checked", is to be placed in

databases shortly after.

In line with its previous policy, the centre says that those receiving the pilot project grants — while free to apply for patents on work involving additional biological experiments that reveals "convincing evidence for utility" of particular sequences (which may include full-length genes) — are also being "discouraged" from applying for patents on the raw genomic sequence themselves.

The NCHGR endorses the principle of patent protection as being necessary for the development by private companies of diagnostic and therapeutic products. But, it says, "patent applications on large blocks of primary human genomic DNA sequence could have a chilling effect on the development of future inventions of useful products". □

Nobel laureate charged with sexual abuse

Washington. Daniel Carleton Gajdusek, a Nobel prizewinner for his work on infectious diseases and a prominent researcher at the National Institutes of Health (NIH), has been charged with sexually abusing a fifteen-year-old boy whom he had brought to the United States from one of his many anthropological research missions to the South Pacific.

Gajdusek, 72, who was arrested on 4 April after an investigation by the Federal Bureau of Investigations, denies the charges. He has brought up to fifty young people back to the United States from New Guinea and Micronesia, caring for them in his home outside Washington DC, and paying for their education.

Several colleagues of Gajdusek expressed shock and disbelief at the charges, and some are said to have helped raise the \$350,000 bail on which

he was released from custody on 6 April.

Gajdusek, who heads a central nervous system laboratory at NIH, won the Nobel prize for medicine in 1976 for his research on mechanisms concerning the origin and dissemination of infectious diseases, including kuru, which afflicts islanders in New Guinea. Much of his work is anthropological, and he has published descriptions of sexual relations between adults and children in primitive societies.

It is understood that these descriptions attracted the interest of the US authorities, who had previously investigated Gajdusek's relationship with the children he had brought back. But charges were filed only with the cooperation of a Micronesian man, now aged 23 and a college student, who claims to have been abused in 1987. **C.M.**

Spotlight turns on blood policy advisers

Tokyo. Now that Japan's haemophiliacs have reached a settlement with the government and pharmaceutical companies over their infection with HIV by blood coagulants (see *Nature* 380, 278; 1996), attention has switched to investigation of possible criminal charges against individuals responsible for setting policy on blood products in the early to mid-1980s.

Shortly before the settlement was reached late last month, the Tokyo District Prosecutor's Office announced that it had set up a team of six prosecutors to investigate possible criminal charges. Chief among those to be investigated is Takeshi Abe, who headed an AIDS study group set up by the ministry in June 1983. Abe advocated the continued use of non-heat-treated concentrates of blood coagulants, and insisted on clinical trials for heat-treated products, a process that under Abe's direction took nearly two years.

Abe's position won the day over those of others in the ministry and the study group who had suggested various alternatives, including emergency imports of heat-treated products, a ban on the imports of non-heat-treated blood products, and a return to the use of cryoprecipitate, a blood coagulant which, unlike concentrates, is obtained from a small number of donors.

Abe has come under severe attack by the Japanese media because, immediately before being appointed head of the AIDS study group, he had started to solicit large donations from blood product manufacturers for a foundation he wanted to set up. There have also been allegations that he continued to seek money from blood product manufacturers while he was coordinating clinical trials of heat-treated products for the companies between 1984 and 1985.

Atsuki Gunji, a former ministry official who set up the AIDS study group, told *Nature* in 1988 that he received "many complaints" about Abe's fund-raising activities. At the time, Abe denied that he had used his influence to delay the introduction of heat-treated products. But there have recently been widespread suggestions in the Japanese media that Abe may have done so, in order to enable Japanese manufacturers which were behind in the development of heat-treated products — in particular Midori Juji (Green Cross Corporation) of Osaka — to catch up with US and European manufacturers.

Abe, who recently resigned as vice president of Teikyo University because of

adverse publicity surrounding him, flatly denies any connection between his fund-raising and his decision-making as head of the AIDS study group and coordinator of the clinical trials of heat-treated products.

The allegations made against him, however, relate to a different matter. In 1994, a group of haemophiliacs filed charges with the Tokyo prosecutor's office of "wilful negligence resulting in death" (see *Nature* 368, 680; 1994). Similarly, earlier this year the mother of a haemophilic patient of Abe's

who had died of AIDS filed charges of murder against him (see *Nature* 379, 664; 1996).

The haemophiliacs claim that Abe continued to administer non-heat-treated products to his patients in 1985, even although he knew there was a high possibility of infection with HIV. As evidence, they point out that, in late 1984, Abe received results from Robert Gallo's laboratory in the United States which showed that 23 out of 48 patients under Abe's care

were already infected with HIV. They also cite a paper published by Abe which shows that some of his patients became HIV-positive only in 1985.

Furthermore, they claim that there is evidence that Abe should have been aware of the dangers long before this. Abe had a haemophilic patient who died of classic AIDS symptoms in July 1983, and this is now widely believed to be Japan's first AIDS patient. But in 1983, Abe's colleagues in the AIDS study group and the Ministry of Health and Welfare did not recognize the patient as an AIDS victim — despite the fact that a US expert had told Abe and ministry officials that the patient was almost certainly an AIDS sufferer.

When the allegations were made in 1994, Abe refused to comment on them, saying he had been instructed to remain silent by the prosecutors' office. The office is also planning to investigate an accusation of perjury that has been filed by a group of haemophiliacs against Gunji. They claim that Gunji gave false testimony in the Tokyo court case when he had said that he was unaware of the similarity of infection routes for HIV and hepatitis B.

The Ministry of Health and Welfare recently released another report on its internal investigation into the blood scandal. But the report has only created confusion, rather than clarifying the decision-making process that took place in 1983.

Three members of the AIDS study group

told ministry officials they remember that, at the time the study group was set up in June 1983, Gunji suggested that heat-treated products could be imported on an emergency basis. In particular, Yuichi Shiokawa, who subsequently headed the ministry's AIDS committee after Abe, says he was surprised by Gunji's suggestion because it showed how seriously the ministry was viewing the matter.

But other members of the study group, including Abe, say they have no recollection of Gunji suggesting emergency imports. And Gunji himself says that emergency imports were only informally discussed by ministry officials as one of many options, and were not formally considered by the study group.

However, other documents released by the ministry show that a subcommittee of the AIDS study group, headed by Mutsuyoshi Kazama of Teikyo University, discussed emergency imports of heat-treated products at its first meeting on 14 September 1983, but dismissed the idea and insisted on clinical trials of heat-treated products. According to Kazama's notes of the meeting, Gunji also asked them to consider a reversion to use of cryoprecipitate. But this idea was also rejected.

The ministry has made no attempt to determine who is telling the truth about the discussion of emergency imports, nor has it provided an explanation of why the idea was dropped. Rather, it has simply released a set of contradictory statements. Haemophiliacs are now calling for an independent investigation, by, for example, the Japanese Diet (parliament).

In addition to the accusations against Abe and Gunji, prosecutors in Osaka will investigate a charge of murder that has been filed against a former president of Green Cross Corporation. The accusation has been made by the relatives of a non-haemophilic who died of AIDS after receiving non-heat-treated blood products in April 1986 for treatment of liver disease.

They claim that Renzo Matsushita, president of the company for five years after heading the ministry of health's Pharmaceutical Bureau, "shipped the products for profits, knowing that patients who received the products will contract HIV and die".

During the course of the ministry's investigations of the blood scandal, it has been established that Green Cross Corporation gave false statements to the ministry about the time of its withdrawal of non-heat-treated products.

The company claimed to have withdrawn all non-heat-treated products by May 1986, but it did not in fact complete withdrawal until April 1988. Furthermore, it continued to supply non-heat-treated products to hospitals well into 1986, months after it claimed to have stopped.

David Swinbanks



Abe: remaining silent on haemophiliacs' accusations

South African academy in search of funds and a role

Pretoria. After a long period of gestation, South Africa's new national academy, embracing both the natural sciences and humanities, was formally launched by President Nelson Mandela last month. The academy now faces the task of raising funds for activities — and deciding what role it will play in national debates on science, technology and education policy.

The new body, the Academy of Science of South Africa, will be based in Pretoria, and has the support of the three existing academies: the predominantly English-speaking Royal Society of South Africa, the Afrikaans-speaking Suid-Afrikaanse Akademie vir Wetenskap en Kuns and the Science and Engineering Academy of South Africa (SEASA).

But the new body is not an amalgamation of these academies. The two latter institutions will continue to focus on their own sectional interests, the first being concerned primarily with promoting the use of Afrikaans as a language, and the second with fostering science and engineering in black communities.

In terms of its stated aims, the main potential overlap is with the Royal Society of South Africa. The president of the latter organization, George Ellis, says that the society is reserving judgement about its future until the new academy has defined its role more clearly.

The new academy differs from the Royal Society of South Africa in that it embraces the humanities as well as the sciences. The society is also perceived by some observers as being regionally based (in Cape Town) and having an excessively narrow base, as its members and fellows are mainly English-speaking whites.

The president of the new academy, Khotso Mokhele, a microbiologist who is the new head of the Foundation for Research Development, is keen that its role should be that of an "activist", promoting scientific thought in a broad sense. "This is not a tea and cookie society," says Mokhele. "The academy needs to make its mark in determining and implementing policy."

Reflecting this fact, the 96 founder members are made up not only of scholars who have made outstanding contributions to research, but also senior civil servants and university and research council administrators perceived to be in a position to promote the academy's objectives. In this sense the body differs from most academies in other countries.

At the same time, there are some glaring omissions in its membership, particularly in the humanities, including the social historians Charles van Onselen and Colin Bundy

and the author and critic John Coetzee. These result from the initial selection process, which was controlled by the three old academies, each of which has a different vision of what an academy should be (see *Nature* 361, 198; 1993).

Furthermore, funding prospects remain uncertain. Mokhele says that the academy needs to attract a diversity of funding sources from both the government and private sectors in order to maintain its independence. He points out that in other countries, national academies derive their income from various sources, ranging from direct government grants through contract research to (in the case of Australia) the sale of textbooks. As yet, the government of national unity has promised no funding.

At the academy's first meeting last month, members discussed various possible interventions in the fields of education and health, to be considered by its first council. For example, it was felt that the academy might become involved in the debate on a new national health plan.

Curriculum reform in the primary and secondary sectors was also suggested, as well as the possible establishment of a national qualifications framework. A further proposal was that the academy should become involved in assessing the research performance of university departments, if the National Commission on Higher Education decides to include such measures as part of a new funding formula for universities.

Michael Cherry

UK Parliament gives approval to terms of chemical arms ban

London. Britain's ratification of the Chemical Weapons Convention passed its final hurdle last week when it was approved in a third reading by the House of Lords, and has now been signed into law.

Although the government has not accepted demands for the creation of a statutory advisory body, as it had been urged to do by those who are keen to see a source of independent advice (see *Nature* 379, 479; 1996), it has promised that a non-statutory advisory mechanism will be established in the near future.

The government has also pledged to publish details of a proposed appeals process for researchers who are denied a license to use particular chemicals. Furthermore, in response to the claim that many of those in the academic community engaged in chemical research are still unaware of the extent to which the convention may affect their activities, the government has said that it will take action to ensure that the new bill is publicized as widely as possible.

Full implementation of the convention will require its ratification by 65 member states. It is widely hoped that this number will be reached by the end of June, allowing the convention to come into force at the beginning of next year.

In the United States, the treaty has already been passed out of the committee stage of the Senate, and ratification is now seen as largely a formality, primarily awaiting a decision by Republican leaders to schedule a debate on the Senate floor. □

New laser is 'the best and brightest'

London. Last week saw the first pulses fired in the world's most powerful ultra-violet laser, Titania, situated at Britain's Rutherford Appleton Laboratory (RAL). Based on two decades of development of krypton fluoride lasers at RAL, the new laser will be able to subject samples placed inside a target area (see right) to single pulses of up to 10 terawatts (10^{12} watts).

Titania will be used for a range of research applications that include fundamental plasma physics, the development of X-ray lasers, and various aspects of inertial confinement fusion. It will replace RAL's Sprite laser, which had been operating as a central facility for researchers from both Britain and abroad since 1981.

Inaugurating the laser, Paul Williams,

IMAGE
UNAMAGABLE
FOR A CORN LABET
FOR A CORN LABET
REASONS

chief executive of the Council for the Central Laboratory of the Research Councils — which is now responsible for RAL — said that the funding restrictions under which the RAL's laser facilities had been built up over the past 20 years meant that its development teams "have had to be clever where others have just used raw dollars." □

NASA to teach 'epidemiology by satellite'

Boston. The US National Aeronautics and Space Administration (NASA) is about to welcome the first researchers and health officials from developing countries to a programme teaching them to use data from satellites and remote-sensing technology to combat infectious diseases transmitted by insects, rodents and other vectors.

This concept, called 'landscape epidemiology', is based on the analysis of data from Earth-imaging satellites to detect changes in the habitat of disease carriers which may give early warning of impending epidemics.

No new satellites will be needed; instead, scientists can draw on data from existing satellites such as Landsat. Ultimately, some NASA researchers hope, it may be possible to generate 'risk maps' showing the risk of contracting a particular disease on a given day anywhere in the world.

"There may be a new way of fighting diseases by monitoring the risk of infection which, in turn, depends on understanding the ecology of the organism that spreads the disease," says David Peterson, a scientist at the NASA Ames Research Center in Moffett Field, California.

The potential of this approach was described at a conference in Baltimore, Maryland, last November. The event, spon-

sored by NASA and the Third World Foundation, was attended by health ministers and medical directors from more than 20 countries, including Bangladesh, Belize, China, Ghana, Indonesia, Kenya, Malaysia, Nigeria, Peru and Rwanda. A training programme for scientists from developing countries, which was announced at the conference, is due to begin shortly.

The goal of using satellite data to reduce the spread of vector-borne diseases is based on the results of NASA-sponsored research during the past decade. Satellite images enabled scientists, for example, to spot mosquito breeding-grounds in rice fields near Sacramento, California. They found that 15 per cent of the fields were home to 90 per cent of the mosquitoes.

In Mexico, scientists were able to predict which villages were likely to have the greatest malaria problems on the basis of the presence of two landscape features: a pasture with cows or horses near the village, and a transitional swamp. A study in New York state also identified specific 'landscape elements' strongly correlated with the incidence of Lyme disease.

Using satellites in this way provides an efficient way of locating disease 'hot spots' and 'danger zones', says Maurice Averner,

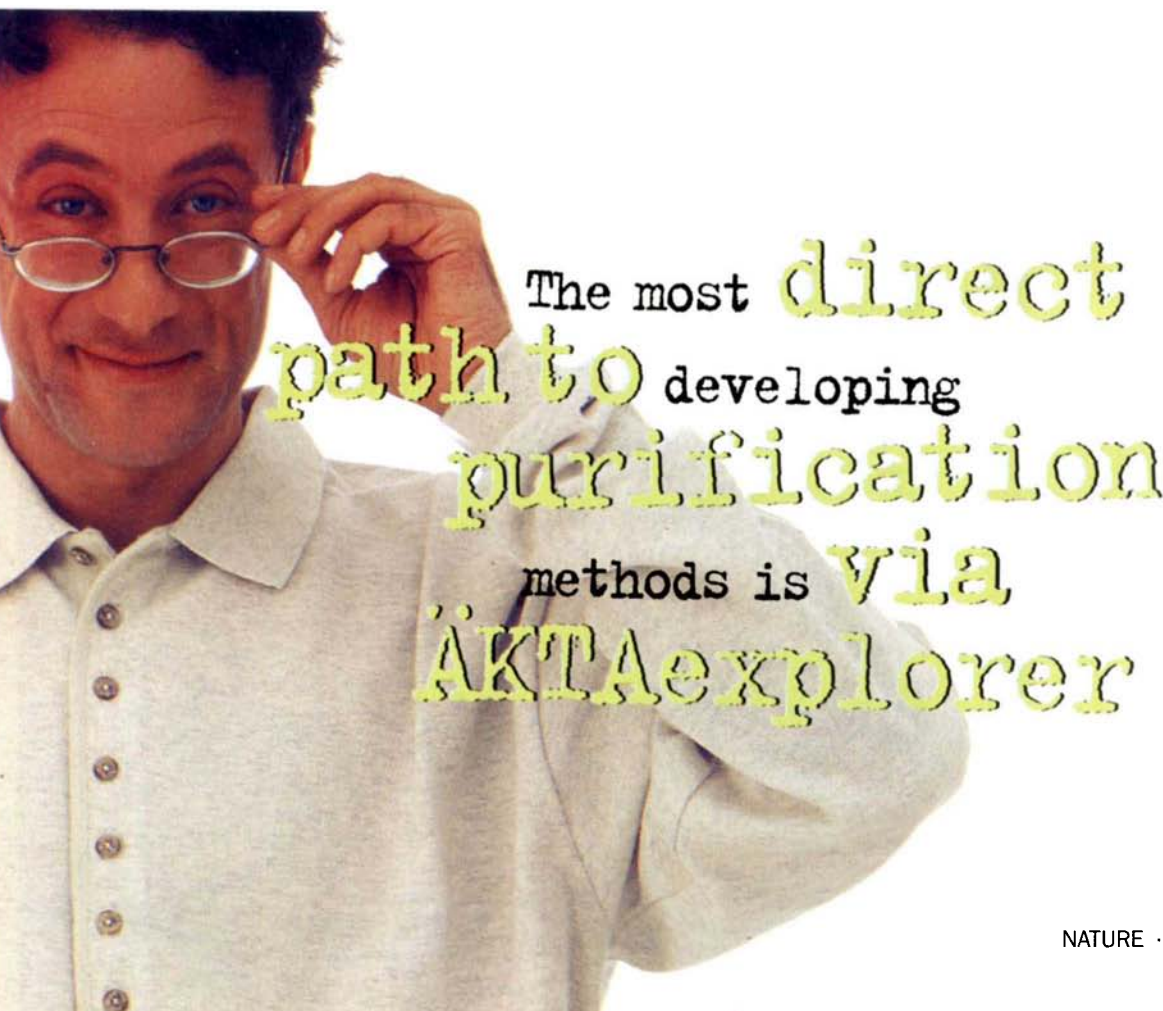
NASA's programme manager for Global Monitoring and Disease Prevention. "Mosquitoes don't breed randomly and indiscriminately. If you can find out what sites they like, you could spray pesticides there at just the right time. That's safer and more cost-effective than spraying everywhere."

The technology can help governments to focus attention on those areas posing the greatest risks, adds Peterson. "It can help us determine when certain medicines will be needed in various places. Based on the information we receive from satellites, we might even consider moving people out of an area temporarily, if the risk of infection is particularly high."

NASA is now planning a series of workshops to inform health officials about the applications of Earth-imaging satellite data to specific diseases such as malaria and dengue fever. NASA Ames specialists will collaborate with researchers from the National Institutes of Health and the Centers for Disease Control on epidemiological studies involving satellite images.

NASA investigators believe satellite images could eventually aid the fight against cholera, encephalitis, hantaan virus, leishmaniasis, Chagas disease, and schistosomiasis.

Steve Nadis



Misconduct meeting fails to draw sceptics

Washington. A conference on scientific misconduct, which was to have included addresses from Donna Shalala, the health secretary, and many leading authorities on misconduct, has been cancelled after only 18 people registered for it.

The meeting, on "The conduct of science: Keeping the faith", was to have been held at the beginning of May at Keystone, Colorado, where the non-profit Keystone Symposia holds its popular and highly regarded meetings on the progress of specific scientific disciplines or subdisciplines.

"We've never [previously] cancelled a meeting in our 22-year history," says Jim Bennett, director of Keystone Symposia and a professor of pharmacology at Michigan State University. The meeting had been publicized through 103,000 standard Keystone leaflets, an advertisement in *The Scientist*, and detailed programmes sent to the 6,000 subscribers to a newsletter put out by the Department of Health and Human Services' Office of Research Integrity.

Bennett says he did not expect scientists to flock to a meeting whose costs would not have been covered by their grants. But he had hoped that scientific misconduct officers from some of the 800 research universities in the United States, as well as

administrators from science funding agencies, would pay the \$175 fee, enabling Keystone to pay the expenses of the 20 speakers.

The meeting's programme had focused on a report on scientific conduct sent to Shalala and the US Congress last autumn by a commission chaired by Kenneth Ryan of the Brigham & Women's Hospital, Boston. Critics of the report say that hostility to its findings may have caused the meeting to fail.

"If the community felt that Ryan was

going to be implemented, there would probably have been much more interest," says Sam Silverstein of Columbia University, New York, who was due to speak. He says that what happened "may reflect the status of the Ryan report, which has been disavowed by most interested parties".

Ryan, who would have opened the meeting, was not available for comment. His report has been attacked for proposing a broad definition of sanctionable misconduct, and for embracing topics such as animal abuse and the mishandling of radionuclides, rather than confining itself to what are widely viewed as the 'three deadly sins' of fabrication, plagiarism and falsification.

But Michael Zigmond, a neuroscientist at the University of Pittsburgh who would have addressed the forum on instilling good conduct through education, says that he will miss the chance for the two sides of the debate to confront each other. "If they had asked me, I would have said that very few people would come," says Zigmond. "Most people don't feel that they are experts, and believe that it is not going to effect their own science. But this does not mean that scientists don't care about ethics."

Colin Macilwain



If you've ever developed a purification strategy by yourself, you know there is a lot to consider. Creating media screening schemes; designing buffer preparation routines; selecting which columns to use—even listing the tasks takes careful consideration. But now there's a better way of working.

Introducing ÄKTAexplorer

Turn ÄKTA™explorer on, choose a protocol, check running parameters and press start. ÄKTA-explorer does the rest.

Designed for all chromatographic techniques, this new purification system offers you pre-set protocols for every major purification task—including method scouting and media



screening. You'll save time as the system automatically recommends the best columns for your runs. You get fast pH screening as it automatically prepares your buffers from stock solutions. The moment you turn ÄKTAexplorer on, you're presented with a direct path to full-scale purification.

That path is UNICORN®—ÄKTAexplorer's control software. Your scale-up is simplified as the system's software can transfer and implement your methods on purification systems at all scales.

Of course, these are just a few of ÄKTAexplorer's features. So call us at 1 (800) 526 3593 from North America, +81 (0)3 3492 6949 from Japan or +46 (0)18 16 50 11 from the rest of the world (or meet us on the Internet at <http://www.biotech.pharmacia.se/akta.htm>).

**Pharmacia
Biotech**

Uppsala, Sweden. (And the rest of the world)

Brown's death leaves hole in Clinton's technology team

Washington. The death of Ron Brown, the US commerce secretary, in a plane crash in Croatia last week has deprived the Clinton administration of a powerful advocate for both its environmental and technology programmes.

Staff at the National Oceanic and Atmospheric Administration and the National Institutes of Standards and Technology — the science agencies which between them comprise the larger part of the Department of Commerce — had come to admire his energetic fight to defend their budgets, as well as the department's continued existence, and were said to be distraught at the news of his death.

In particular, Brown had enthusiastically backed the Advanced Technology Program, seen as a key mechanism for using federal funds to stimulate industrial innovation. Brown's duties have been temporarily assumed by Mary Good, the equally indefatigable under secretary for technology in the commerce department. □

Japan turns on neutrino detector

Tokyo. The world's largest neutrino detector, the Super-Kamiokande observatory, began operations last week 1 km underground in a lead-zinc mine in Gifu prefecture in central Japan. Super-Kamiokande is more than ten times the size of its predecessor Kamiokande in the same mine, which in 1987 detected neutrinos from a supernova in the Large Magellanic Cloud.

The new detector, which cost more than ¥10.4 billion (US\$100 million) to build, consists of a tank of crystal clear water 40 metres wide and 40 metres deep. It is lined with 11,200 highly sensitive 20-inch photomultiplier tubes that can pick up very faint Cerenkov radiation when neutrinos collide with water molecules. The detector is located deep underground to filter out cosmic radiation. □

Chernobyl 'cancers' ruled out...

London. A Scottish health board has ruled out a local doctor's claims that a rise in the numbers of cases of cancer on a Scottish island could be linked to the Chernobyl nuclear accident of 1986.

Francis Tierney, a general practitioner on Benbecula in the Outer Hebrides, said he had diagnosed 18 cancers in a 19-month period and suggested that fallout from the Chernobyl accident could be one possible cause.

The claims made headline news in the British media. But last week the Western Isles Health Board said one cancer every month was not unusual for that region and that no cancer had been induced by radiation. Officials said, however, that a complete analysis would take a further three weeks. □

...as Ukrainians fear a repeat

London. One-third of Ukrainians polled from government, parliament, industry, universities and the media believe that a Chernobyl-style nuclear accident could be repeated.

The respondents, who took part in a survey shortly before the accident's tenth anniversary, blame poor management, and believe that the chances of a second accident are higher than 50 per cent. Fifteen of the so-called RBMK graphite-moderated reactors still operate in countries of the former Soviet Union. □

UK physicists get greater role

London. Britain's Institute of Physics has reached agreement with the Engineering and Physical Sciences Research Council (EPSRC) on how its members should play a greater role in discussions of the council's overall research policy. Some physicists had expressed concern that they were not consulted before the council's recent decision to cut back its funding for physics research while increas-

ing that for both mathematics and chemistry (see *Nature* 379, 755; 1996).

In particular, the EPSRC has agreed to invite the institute to comment annually on the business plan for the physics programme, while the institute has agreed to bring 'burgeoning areas of physics' to the attention of the council. □

Maths PhD 'may be Unabomber'

Washington. Theodore Kaczynski, a 53-year-old former mathematics professor, was last week taken into custody by the US Federal Bureau of Investigation (FBI), suspected of waging an 18-year campaign to kill scientists under the name of 'Unabomber'.

Kaczynski taught mathematics at the University of California at Berkeley for two years, after taking his PhD there in 1967. He was betrayed to the police by suspicious family members. FBI officers kept watch outside the wooden hut in Montana where he has lived since resigning his post at Berkeley in 1969. Unabomber, who killed three people and wounded a further 23 during his campaign, was opposed to modern technology. He frequently penned lengthy documents setting out his views. □

Chemists take over their lab

London. The UK Laboratory of the Government Chemist (LGC) has been taken over by a management-led consortium as part of the privatization of all research laboratories owned and operated by the Department of Trade and Industry.

The laboratory was sold to LGC Holdings, a company formed by a consortium led by Richard Worswick, the Government Chemist, and including LGC management and staff, as well as the Royal Society of Chemistry and the investment group 3i. Worswick remains Government Chemist. □

WHO seeks 'minimal' BSE risks

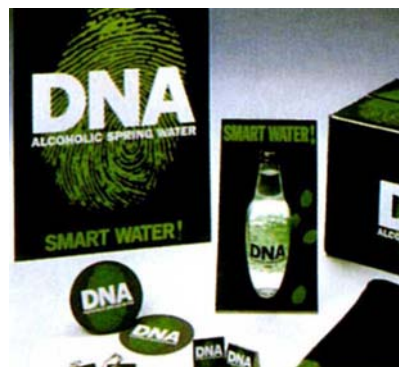
London. A two-day meeting at the World Health Organization of scientists engaged in research on spongiform encephalopathies concluded that the risk of bovine spongiform encephalopathy (BSE) being passed to humans would be "minimal" if their recommendations were implemented. The recommendations included banning from the human and animal food chain all parts of a cow that has shown possible signs of BSE, as well as animal brains, spinal cords and eye retinas. □

DNA heads for the drinks cupboard

London. International regulatory authorities have allowed a private company in South Australia to profit from the sale of DNA. The material is packed in fluorescent green bottles and is available at supermarkets, petrol filling-stations, even nightclubs, priced at just US\$1.50 for 350 millilitres.

Lawyers and human rights groups have so far not reacted, possibly because the colourless liquid in question is a new brand of alcoholic spring water. Advertising campaigns for the drink — a wine-based beverage containing 5.5 per cent alcohol by volume and flavoured with lime and thyme — bear the message: "Where there's life, there's DNA".

"Everybody who sees it, just cracks up," says Islay Kennedy, proprietor of Vickery Wines, which holds the rights to the drink in the United Kingdom, where it will be launched next month. □



Try, try, and try again

SIR — Bill Amos warns that the accepted standard approach to statistics can easily lead to erroneous results (*Nature* 379, 484; 1996). This is because, over the whole world, many scientists may be studying the same phenomenon or closely related phenomena, and several different scientists may perform the same experiment or a similar experiment unknown to each other. Even if the experiment should show no correlation at all, as more scientists try, the chances of one of them producing a 'statistically significant' (and erroneous) result quickly rise.

Although Amos states the case well, he cites numbers of investigators that might seem large enough to make such happenings unlikely or even unrealistic. Actually, the way science operates plays so directly into this statistical trap that large numbers of unwitting participants are not required. In science, failures are very rarely mentioned. Thus, if an experiment is a logical next step, the idea will naturally occur to scientists in its field. One after another will pursue it until one of them succeeds and is published. If after publication another scientist tries the same experiment and fails to replicate it, that typically will go unreported because such a report is very difficult to get past the referees and might tarnish the author's reputation in the process. Usually it is only when a result proves a major stumbling-block to continuing research that any real effort is made to unseat it. Much more often, if a scientist finds that a result cannot be replicated, then a way is found to sidestep it.

The most important effect of most studies (erroneous or not) is that they influence future work, directly or indirectly. As scientists ponder what to do next, the erroneous study will influence some scientists as they design experiments in parallel courses and other scientists as they design experiments in other areas that seem logically related. If more than thirteen experimental trials which should show no correlation are influenced by the erroneous study, the chances are better than even that at least one will show $P < 0.05$. The cycle continues. And, those experiments that do not show significant results will go unreported and so will still seem a logical course to other scientists, waiting to be tried again. Some of these re-tried experiments show statistical significance....

This becomes much more alarming when it is realized that this description of scientific practice is modelled very closely by a now classic combinatorial probability game in which a black ball and a white ball are placed in an urn (R. M. May, *Nature* 262, 646; 1976). If one draws a white ball, then that ball is replaced and another white ball is added to the urn for future draws. If one

draws a black ball, it is replaced and a black ball added to the urn. The game continues with successive draws and is scored by taking the ratio of white balls to black balls in the urn on successive runs. Thus the result of each draw or 'experiment' influences the result of future experiments.

If one tries this game, one finds that the ratio quickly stabilizes to a steady value of 0.674... and is quite deterministic in settling to that result. One might think this result strange, but it is easily explained when one understands that the surface texture of the two colours of ball are slightly different, probably influencing the draw. With this simple game, however, one has the distinct advantage of being able to start again from the beginning, with one black and one white ball. The ratio will again quickly stabilize in a quite deterministic manner. It will, however, stabilize to a completely different value, say 0.228... The surface texture explanation is meaningless. Each time the game is played, it appears as deterministic as any chain of scientific studies, but each time the final deterministic-appearing result is different.

This same mechanism, caused by the inevitable interaction of statistics and standard scientific practice, will also lead to deterministic-appearing chains of scientific development which are quite false. There do not have to be large numbers of scientists doing the same experiment for this to happen.

David Dunthorn

C F Systems,
908 West Outer Drive,
Oak Ridge, Tennessee 37830, USA

SIR — Amos illustrates in a fictitious scenario how chance might lead to reporting of a non-existent 'finding' (type 1 error). He appears to have used one-tailed tests throughout his illustration and to have overlooked the probability of a similar distribution of his 50 'significant' (at $P < 0.05$) findings among 1,000 trials in both tails of the distribution. If the spurious 'findings' were in truth nonexistent, the 1,000 trials would yield only some 25 'significant' positive reports balanced by some 25 'significant' negative reports (and 950 non-significant outcomes supporting the 'null hypothesis'). Publication of an unbiased mix of 'significant negatives' and 'significant positives' would end the illustration there.

Although truly 'negative' findings (not 'null' findings) may be as important to science as 'positive' findings, scientists often seek trends in one direction, which one depending on the field of enquiry and the current consensus paradigm. Publication bias may distort the fair finding of 25 positive trials and 25 negative trials by selective-

ly reporting more of those that show an association in one direction (perhaps the 'positive'). It is not the play of chance that inhibits scientific advance so much as the play of prior prejudice, seeking significant findings in only one tail of the distribution.

Robert West

University of Wales
College of Medicine,
Heath Park, Cardiff CF4 4XN, UK

Legal pill

SIR — In their Commentary article "Why Japan ought to legalize the pill" (*Nature* 379, 579–580; 1996), Maruyama *et al.* say that "Japan is the only industrialized country, except for the Republic of Ireland, where steroid oral contraceptives are illegal". That is not correct. Steroid oral contraceptives have been available for prescription in Ireland for more than 15 years, and the range of such products available is comparable to that in any other European country.

John G. Kelly

Irish Medicines Board,
63-64 Adelaide Road,
Dublin 2, Ireland

In the ears of the beholder?

SIR — As Hunt and Balsan have pointed out¹ musicians have many beliefs about violin tone, but it was established long ago that hearing cannot distinguish between the bowed sounds of old and new violins².

High damping is desirable in spruce³ and in strings⁴. Wolf notes are due to low damping. When a violin correctly set up at 60–70 per cent relative humidity is exposed to the dry air of a centrally heated atmosphere, it changes shape and, for example, the gap between strings and fingerboard may be reduced so as to make it unplayable; in comparison, any changes in the physical properties of the wood are trivial.

If playing or age changes wood or other vital components such as glue, a skilled player compensates for the properties of each individual instrument, and there is no way in which the effect of any property can be detected by a listener⁵. Most of the beliefs about violin tone appear to be subjective.

James Beament

Queens' College,
Cambridge CB3 9ET, UK

- Hunt, D. G. & Balsan, E. *Nature* 379, 681 (1996).
- Saunders, F. A. J. *Frank. Inst.* 229, 1–20 (1940).
- Cremer, L. *The Physics of the Violin* (MIT Press, 1985).
- Pickering, N. C. *The Bowed String* (Amerion, New York, 1989).
- Beament, J. *The Violin Explained* (Oxford University Press, in the press).

Is global warming climate change? Hungarian science

SIR — Hardly had 1996 arrived when the UK Met. Office issued a press release to the effect that 1995 was the warmest year so far¹. But the press release failed to contain three important provisos.

(1) The December surface observations were not yet available, so that provisional information from upper air charts was used to compute an educated guess. The December temperature anomaly used was therefore not compatible with the other months of the year.

(2) The temperature anomaly for the globe was determined largely by that for the Northern Hemisphere. The Southern Hemisphere had nowhere near the warmest year so far, even if computed from surface data.

(3) The NASA MSU satellite record which monitors the temperature of the atmosphere showed that 1995 was eighth out of the 17 years available.

Interestingly, the fall in temperature in the Northern Hemisphere from November to December was more than 0.7 °C, constituting the greatest month-to-month change in the entire record of 203 values.

In the context of the global warming debate it is important to define what is meant by the term 'climate change'. The Australian Delegation to the Madrid meeting of Working Group 1 of the International Panel on Climate Change (IPCC) succinctly pointed out a prevalent ambiguity.

"Climate Change" in IPCC Working Group 1 usage refers to any change in climate over time whether due to natural variability or as a result of human activity. This differs from the usage in the Framework Convention on Climate change where 'climate change' refers to a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and which is in addition to natural climate variability observed over comparable time periods.²

Let us consider the first definition and ask whether natural variability can produce long-term trends comparable to those observed in the historical record and those predicted to occur during the next century.

During the past ten years, it has often been stated that the previous year was the warmest on record, or that the past decade contains six of the warmest years on record³. Such statistical events may not constitute as clear an indication of induced global warming as might appear at first sight. There is an alternative explanation, the random walk. The somewhat remarkable mathematical properties of the simple random walk resulting from games of chance are well described by Feller⁴. One of these is that the winning player is likely to be further ahead at the end of the last trial than at any previous time. This property alone is conducive to the generation of trends in games of relatively short

duration. Analogies between the statistical properties of random walks and temperature trends have been made by several authors^{5,6}.

Bye^{7,8} introduced the concept of 'bounded random walks', the extents of which increase as the exchange coefficient with the deep ocean decreases. It is suggested that there are two processes determining a climate which is not being subjected to human interference. One is a cyclic solution representing a deterministic system in which all properties can be forecast from the radiation cycle; the other is due to perturbations in the radiative and ocean current fields, which give rise to bounded random walks, which are superimposed on the former to produce the observed interannual climate variability. Although it is not the intention of the authors to deny that the observed trends in the surface temperature record may be caused by human interference (the enhanced greenhouse effect), it is proposed that the observed variability, including any observed trends, may be explained by internal mechanisms which drive the ocean-atmosphere system.

The IPCC has given little or no consideration to the generation of 'bounded random walks' in their publications and pronouncements.

Adrian H. Gordon

John A. T. Bye

Roland A.D. Byron-Scott

Flinders Institute of

*Atmospheric and Marine Science,
Flinders University of South Australia,
Bedford Park, 5042,
South Australia*

1. Meteorological Office press release (1996).
2. WMO-UNEP IPCC Working Group 1 Fifth Session, Madrid 27–29 November 1995.
3. Ratcliffe, R. A. S. *Weather* **2**, 193–194 (1990).
4. Feller, W. *An Introduction to Probability Theory and its Applications* Vol. 1, 77–87 (Wiley, New York, 1960).
5. Woodward, W. A. & Gray, H. I. *J. Clim.* **8**, 1929–1937 (1995).
6. Gordon, A. H. & Bye, J. A. T. *Glob. planet. Change* **8**, 181–188 (1993).
7. Bye, J. A. T. *Earth Sci. Rev.* (in the press).
8. Bye, J. A. T., Byron-Scott, R. A. D. & Gordon, A. H. *J. Clim.* (in the press).

Yes to transplants

SIR — Paula J. Mohacs et al. quote me as having said, in a personal communication, that opposition to xenotransplantation is expressed by, among others, potential transplant recipients (*Nature* **378**, 434; 1995). The facts are that 10 per cent of such patients expressed opposition, 90 per cent did not. The 90 per cent was made up of 50 per cent of patients in agreement and 40 per cent who were undecided.

Ian F. C. McKenzie

*Austin Research Institute,
Studley Road, Heidelberg,
Victoria, Australia 3084*

SIR — *Nature* recently published a report about Hungarian science seeking guidance from the European Union¹. We agree that international cooperation is needed, but we would like to add a comment.

The Hungarian government started a new system for the support of research in 1986, the first country in the area to do so. That system, like those of most industrialized countries, is based on applications and grants, independent of government regulations and the current political situation.

The applications are first submitted to the OTKA (State Research Foundation), which asks for the opinion of three independent and recognized experts in the field. Second, an expert committee will rank the applications. Grants are for between one and four years, and progress is checked periodically. The system seems to work efficiently, to judge by the *Science Citation Index*², which shows that Hungarian scientists are approximately as productive as their colleagues in countries with a wealthier funding background. For instance, in the most recent academic period for which there are figures, researchers at the institution at which we

	Number of projects	Support in HUF
Social sciences	731,409,000	498
Natural sciences	1,553,132,000	697
Life sciences	1,292,276,000	528
Total	3,576,817,000	1,723

work, which is Hungary's largest medical university, published 2,406 articles with a cumulative impact factor of 1846.997.

The system is certainly functioning well, but some features cause concern. For example, well recognized and successful centres (sometimes understandably) claim for more support. This means that new teams or researchers that are not yet well known may face difficulties in finding grants to support their projects. Another problem is that while Hungary's gross domestic product (GDP) has been growing, the funds for research have been decreasing, not only in total value, but also in percentage of GDP (2.6 per cent in 1988, compared with 0.7 per cent in 1995). Finally, although the role of private foundations, both domestic and foreign, has been growing, it has not yet reached the importance of OTKA.

The table shows projects supported by the OTKA in Hungary, for the next academic period³. (US\$1 = 136 Hungarian forints.)

Zoltan Vajo

Bela Szekacs

*II. Department of Medicine,
Semmelweis University,
Budapest 1008,
Hungary*

1. *Nature* **379**, 672 (1996).
2. Schubert, A., Glangel, V., Braun, T. (eds) *Scientometric Data Files* (Hungarian Academy of Sciences Press, Budapest, Hungary, 1992).
3. Tamasi P. (ed.) *OTKA News* (Hungarian Academy of Sciences Press, Budapest, Hungary, 1995).

Novel sensations in the congenitally blind

Tim Pons

A report on page 526 of this issue shows that, in blind subjects, the primary visual cortex becomes activated via the neural pathways mediating the sensation of touch. The implications of this finding are many and various.

How does the brain process sensory information so that it results in the sensation of vision or touch? Most neuroscientists would not be so bold as to claim they could fully explain the neural basis of sensation, but they would probably offer the following partial explanation.

Information for vision and touch is relayed from the eye or skin to the brain along physically separate pathways¹. At the level of the cerebral cortex, the highest level of processing in the brain, there are separate 'primary' areas that process information independently for vision and touch. These areas are commonly referred to as the primary visual, and primary somatosensory, cortex (see figure). Although additional processing and even a merging of visual and somatosensory information takes place in higher order cortical areas, such processing is dependent upon the primary cortical areas^{1,2}. The independence of the visual and tactual pathways at the earliest stages of cortical processing is perhaps best exemplified by the fact that a loss of primary visual cortex results in blindness but does not cause a sensory impairment in touch. Likewise, loss of primary somatosensory cortex results in a loss of tactual discrimination and not in defective vision.

What happens to the neural circuitry in the primary visual cortex of a blind person (or animal)? The question has been addressed through studies of the various causes of blindness (for example severed optic nerve versus visual deprivation) in one or two eyes, and at different stages of development (early childhood versus adulthood) (see ref. 3 for review). A widely held view to emerge from such experiments is that there is a 'critical period', or brief window of opportunity, early in childhood, during which major alterations in primary visual cortex can take place after a given eye manipulation. Importantly, however, any changes in primary visual cortex that take place during the critical period were thought to be limited to the visual modality. After the

critical period, no type of experimentally induced blindness was thought to result in major changes in the primary visual cortex.

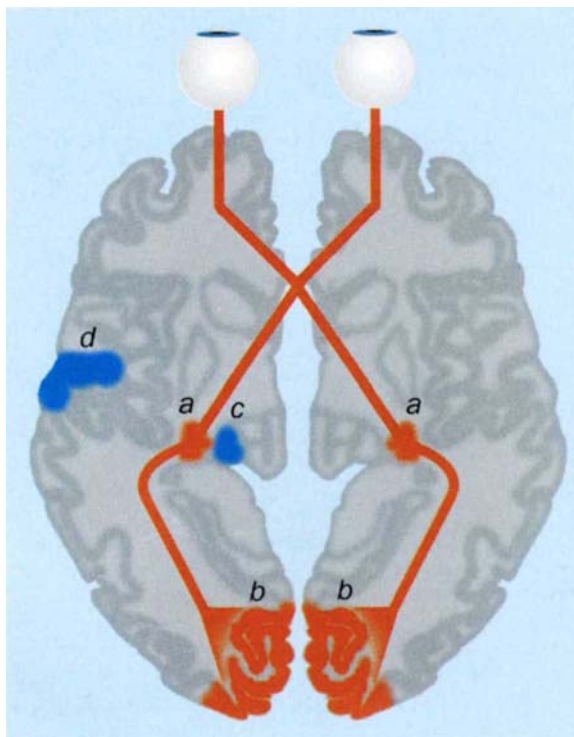
The results reported by Sadato *et al.* in this issue⁴ run counter to this belief, for they show that tactual activation of pri-

senses, such as hearing or touch, though this has been a subject of intense debate³. The findings reported by Sadato *et al.* point to a neurobiological basis for such putative compensatory sensory changes in blind human subjects who perform various tactual tasks, including reading of Braille.

Specifically, the authors measured regional cerebral blood flow, an index of neural activity, from the primary visual cortex (area 17 or V1) of sighted or blind humans while the subjects actively discriminated between tactually distinct objects, including Braille dots, or while they were passively stimulated by the tactual stimuli. Five different types of conditions employing tactual stimuli were used in the study. One of the conditions did not require discrimination by the subjects; instead the index finger of one hand was swept across a homogeneous pattern of raised Braille dots. In the other four conditions the subjects were required to distinguish actively between the tactually distinct objects (as described in the paper). The subjects were not asked to form mental images of the stimuli.

In the passive stimulation condition, which did not require subjects to discriminate between tactual stimuli, there was no activation of primary visual cortex in sighted or blind individuals. The striking result, however, was seen in each of the four active tactual discrimination conditions: blind subjects showed a statistically significant increase in regional cerebral blood flow bilaterally in the primary visual cortex (that is, in both hemispheres), compared to sighted control subjects who showed a decrease of blood flow in the same region.

As to the mechanism (or circuitry) by which tactual activation of primary visual cortex could occur, the thalamus would be the first possible level of processing where vision and touch information could come together; visual and tactual inputs are completely separated until this stage (for example, retinal projections are physically



Structures involved in visual (red) and somatosensory (blue) processing in the brain. Fibres from the optic nerves take two routes to the visual thalamus, which then relays visual inputs to the primary visual cortex. In contrast to the bilateral input from the eyes, the right side of the body is represented only in the left thalamus and primary somatosensory cortex; the left side of the body is represented in the opposite hemisphere. In the experiments conducted by Sadato *et al.* on blind people, stimulation of the right hand by touch caused bilateral activation of the visual cortex (that is, activation in both hemispheres). This cannot be explained by a spread of connections from the tactually responsive region of the thalamus to the visually responsive region, because the left thalamus projects only to the left cortex and vice versa. Bilateral activation of primary visual cortex by touch is therefore likely to occur through other cortical connections not shown here. a, Visual thalamus; b, primary visual cortex. c, d, Left-hemisphere somatosensory thalamus and primary cortex, respectively; the connections between the latter two are direct but not shown.

mary visual cortex in blind human subjects is possible. A common assumption of lay people, and some scientists alike, is that loss of vision is compensated for by an increase in the acuity of the remaining

separated from the spinal cord). But even in the thalamus, visual and tactual inputs remain segregated, and in my view the tactual activation of the primary visual cortex is almost certain to be mediated by cortical mechanisms. This is not only because of the segregation of visual and tactual inputs before this processing level, but because of the fact that tactual stimulation to the right hand results in the bilateral activation of primary visual cortex (see figure). Only the contralateral side of the body is represented in the thalamus, and the lateral geniculate nucleus (the thalamic relay for information to primary visual cortex) relays inputs only to cortex of the same hemisphere, so cross-modal reorganization at the thalamic level could not possibly explain the bilateral activation of visual cortex to a tactual stimulus delivered to one hand.

The earliest level in the somatosensory processing stream where a bilateral representation of the body surface is seen is in higher order cortical areas such as the insula or posterior parietal cortex⁵. Convergence of bilateral somatosensory inputs with visual inputs is seen in the superior temporal processing region of the brain. Such connections could conceivably mediate the tactual activation of primary visual cortex along feedback connections from these higher order areas, which is very similar to the mechanism proposed by Sadato and colleagues.

Sadato *et al.* suggest that the neural machinery which mediates the tactual activation of primary visual cortex in their blind subjects is compensatory in nature, because their blind subjects are superior to the sighted control group in discriminating between Braille letters or words. Of course, as they recognize, much more work will have to be done before a causal link can be made. Even if tactual responsiveness of visual cortex is linked with an increase in the ability of blind subjects to tactually discriminate between objects (and this has not yet been demonstrated), it may have nothing to do with the reading of Braille words.

What is needed are experiments to dissociate the effects of the ability to make fine tactual discriminations from the reading of words consisting of Braille dot stimuli. Further experiments will also be necessary to reveal the role that practice contributes to reorganization in the visual cortex, as well as to a blind or sighted subject's abilities to make tactual discriminations. It is very difficult to carry out such experiments (for example a sighted control group would have to be persuaded to read Braille daily for a prolonged period), but they are essential if we are to tease apart the contributions of experience on an unperturbed (or normal) central nervous system from that of the effect of blindness. Keeping track of variables such as whether blindness is caused by a specific type of

neurological damage (such as to the optic nerve or retina) or by other non-neurological reasons will also be important.

Regardless of whether the processing of tactual inputs by the primary visual cortex in congenitally blind humans is related to Braille reading or not, the unexpected findings reported by Sadato *et al.* raise other fascinating and important questions related to the processing of sensory information in the human brain. What are the perceptual correlates, if any, of the processing of tactual stimuli by primary visual cortex? Is the perception one of visual white noise? Or of touch? Or does processing of tactual inputs by primary visual cortex in blind people result in a fundamentally 'new' or novel sensation not experienced by sighted individuals? Experiments aimed at addressing these

questions are difficult to perform in both humans and animals^{3,6}. But they are essential if we are to understand the effects that genetics, development and life experience have on the cortical machinery, leading to sensory perception. □

Tim Pons is in the Department of Neurosurgery, Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157-1029, USA.

1. Felleman, D. J. & Van Essen, D. C. *Cereb. Cortex* **1**, 1-47 (1991).
2. Pons, T. P., Garraghty, P. E., Friedman, D. P. & Mishkin, M. *Science* **237**, 417-420 (1987).
3. Rauschecker, J. P. *Trends Neurosci.* **18**, 36-43 (1995).
4. Sadato, N. *et al.* *Nature* **380**, 526-528 (1996).
5. Kaas, J. H. & Pons, T. P. in *Comparative Primate Biology* (eds Steklis, H. P. & Erwin, J.) 421-468 (Liss, New York, 1988).
6. Sur, M., Garraghty, P. E. & Roe, A. W. *Science* **242**, 1437-1441 (1988).

PLATE TECTONICS

The valley that time forgot

Kristin M. M. Rohr

SEDIMENTS 140 million years old lie in part of the Atlantic Ocean where the crust is only a fraction of that age. On page 518 of this issue¹ Bonatti *et al.* report the discovery of Cretaceous rocks in a fracture-zone near the Mid-Atlantic Ridge, where new crust is being manufactured and spreading to the east and west. It has implications for the early breakup of a supercontinent and the formation of the equatorial Atlantic, but, more importantly, this discovery cannot be explained by conventional plate tectonics.

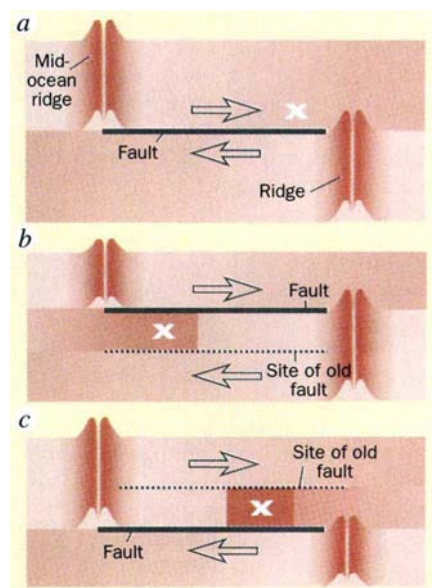
It was thought that the equatorial Atlantic Ocean formed about 120 Myr (million years) ago². But now sediments typical of a continental slope, and fossils 140 Myr old, typical of the ancient North Atlantic Ocean, have been found¹ in several samples recovered from the walls of the Romanche fracture zone that stretches from Africa to South America. A rift basin must already have opened up and been connected to the northern oceans 140 Myr ago.

New oceanic lithosphere is continuously formed at spreading centres, also called mid-ocean ridges, so the age of the lithosphere increases away from these ridges. Segments of the ridge are generally aligned perpendicular to plate motion, and accommodate sinuous or complicated plate-boundary shapes by varying amounts of lateral offset between segments³. The largest offsets are made by transform faults, which are thought to be planes of pure strike-slip movement (sliding parallel to the face of the fault) between two oceanic plates⁴.

How then is a relic from the early opening of the equatorial Atlantic Ocean, preserved in a fracture zone only 135 km

away from the nearest active spreading centre, surrounded by rocks 100 Myr younger?

Bonatti *et al.*¹ question the usual assumption that the structure of the ridge is stable, and propose instead that transform faults jump around. If one were to jump to the north (see figure), lithosphere which had been travelling eastward would now effectively become part of a different plate and move westward, and older



A transform fault separates two oceanic plates that are moving apart at a mid-ocean ridge, allowing segments of the ridge to be offset. Shading indicates age away from the ridges. Repeated jumps of the fault to either side of a slice of crust (b, c) could keep older rocks near to the spreading centre, where conventional tectonics predict the rocks to be young.

material would be set against younger. A later jump to the south could transfer this same chunk of lithosphere back to the eastward moving plate, and a series of jumps could keep old material close to the spreading centre indefinitely. Magnetic anomalies, which usually record plate boundary shifts on the sea floor, are too weak here to show whether this happens. Gradual migrations of transforms that provide small offsets are common enough³, but discrete jumps have never been documented by magnetic anomalies. What would provoke a fracture zone to jump sideways by more than 50 km? There must be great resistance to creating a new fracture zone more than 800 km long in oceanic lithosphere up to 50 Myr old that includes cold, strong material.

Another assumption one could question is that shear motion occurs on a single vertical fault. The Romanche fracture zone, where these rocks were found, defines in part the southern edge of the western bulge of Africa. Geological observations of this continental margin and its counterpart in South America indicate that small basins and ridges were an early part of its development^{5,6}. Within continents, transform plate boundaries can be zones of linked faults which jostle each other in a constant interplay of shear, compressional and extensional forces⁷. In such a zone one can imagine that slices of crust surrounded by active faults are not permanently attached to one plate or another. Thus older rocks might simply be trapped in the fault zone, moving relative to both plates. It is plausible that similar tectonics could occur between oceanic plates.

The notion that transform faults are single planes of pure strike-slip motion stems from early reports of thin zones of freshly deformed rocks in fracture-zone valleys. But refraction studies found that these valleys were underlain by a band of rocks whose seismic velocities are reduced by faulting, brecciation and hydrothermal alteration⁸. If all the motion occurs within a zone 2 km wide, how does a band of crust 6–10 km wide become faulted and altered?

Spectacular seismic reflection data from the sites where the Cretaceous rocks were found provide incontrovertible evidence that the top few kilometres of this 50 by 200 km transverse ridge are sedimentary, and that compressional faults lifted the sediments up to their present position¹. A change in dip and in the character of reflections distinguishes sediments 60 Myr old from those 140 Myr old, and is evidence that previous transpression (strike-slip motion with some compression) was followed by a 100-Myr hiatus of either no sedimentation or erosion, then by sedimentation, and finally by a second episode of transpression. Such a complicated history may be a consequence

of having several active linked faults.

The existence of transverse ridges parallel to some fracture zones has long puzzled Earth scientists. Their morphology is complex and resists generalization^{8,9}. Rocks retrieved from these ridges vary from upper mantle to shallow-water carbonates, and this variability is taken as an indication of the intense vertical tectonism that can occur¹⁰. The origin of the vertical tectonism may be an interplay of forces from the changing relative motions of the plates and the thermal and viscoelastic properties of the oceanic lithosphere. Further north in the Atlantic Ocean, two transverse ridges may have formed because of intraplate extension caused by variable adaptation of plates to changing relative motions¹¹. Are transverse ridges formed by extension? Or compression? Or sometimes one and sometimes the other? Within continents, extension close to compression is a hallmark of deformation distributed over several strike-slip faults. A multiple-fault model of fracture zones can explain the old Romanche sediments without invoking a phenomenon like fault jumping.

Are anomalously old rocks in the Romanche fracture zone an idiosyncrasy of this large offset transform, or are

they important clues prompting us to rethink conventional wisdom regarding oceanic transform boundaries? More detailed mapping and dating of other fracture zones may well yield other sets of interesting structures and anomalously aged rocks. □

Kristin M. M. Rohr is at the Pacific Geoscience Centre, Geological Survey of Canada, PO Box 6000, Sidney, British Columbia, Canada, V8L 4B2.

rohr@pgc.emr.ca

1. Bonatti, E., Ligi, M., Borsetti, A. M., Gasperini, L., Negri, A. & Sartori, R. *Nature* **380**, 518–520 (1996).
2. Nurnberg, D. & Muller, R. D. *Tectonophysics* **191**, 27–53 (1991).
3. MacDonald, K. C., Scheirer, D. S. & Carbotte, S. M. *Science* **253**, 986–994 (1991).
4. Wilson, J. T. *Nature* **207**, 343–347 (1965).
5. Mascle, J. & Blarez, E. *Nature* **326**, 378–381 (1987).
6. Zalan, P. V., Nelson, E. P., Warme, J. E. & Davis, T. L. in *Strike-Slip Deformation, Basin Formation and Sedimentation* (eds Biddle, K. T. & Christie-Blick, N.) 143–158 (Spec. Publ. 37, Soc. of Economic Paleontologists & Mineralogists, 1985).
7. Biddle, K. T. & Christie-Blick, N. (eds) *Strike-Slip Deformation, Basin Formation and Sedimentation* 1–34 (Spec. Publ. 37, Soc. of Economic Paleontologists & Mineralogists, 1985).
8. Detrick, R. S., White, R. S. & Purdy, G. M. *Rev. Geophys.* **31**, 439–458 (1993).
9. Fox, P. J. & Gallo, D. G. *Tectonophysics* **104**, 205–242 (1984).
10. Bonatti, E. *Earth planet. Sci. Lett.* **37**, 369–379 (1978).
11. Pockalny, R. A., Gente, P. & Buck, R. *Geology* **24**, 71–74 (1996).

CELL CYCLE

Why don't plants get cancer?

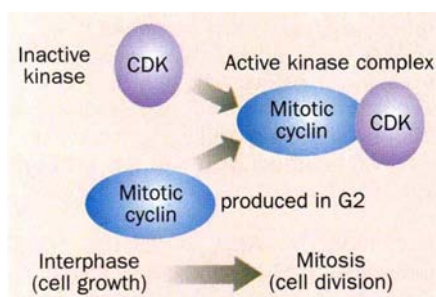
John Doonan and Tim Hunt

EXTRA cells in an animal are dangerous because they usually turn into tumours, but in plants any extra cells can simply be incorporated into normal tissues. In fact, plants as a group are remarkably resistant to neoplastic transformation — the common plant tumours arise only as a result of complex interactions with specialized pathogens such as *Agrobacterium* or gall-forming insects. They are unscathed by their constant exposure to high levels of ionizing radiation in the form of ultraviolet light, which in animals leads to DNA damage and eventually cancer. Such a striking disparity with animals may indicate that this resistance is built into the way plants develop. Although the molecular basis for this apparent difference is undoubtedly multifactorial, a new and intriguing insight into one of those factors is described on page 520 of this issue by Doerner and co-workers¹.

Control of cell growth and division in plants ought, you would think, to be a crucial determinant of the final size and shape of leaves, shoots, stems and roots, whose structures under the microscope record the history of past divisions. But the experiments of Doerner *et al.*¹, in which cell division was stimulated in transgenic plants, and another in which cell division

was impaired², suggest that this is not the case. Indeed, the effect of both manipulations was much the same — within broad limits, increasing or decreasing the number of cells makes surprisingly little difference to how the plant actually looks. True, the plants with extra cells grew somewhat faster than control plants¹, but those with far fewer cells still managed to form a very respectable plant².

Doerner *et al.* managed to increase the number of cells in the roots of their transgenic *Arabidopsis* plants by tampering with the expression of B-cyclin, one of a family of proteins that act in conjunction with a group of phosphorylating enzymes known as CDKs (for cyclin-dependent kinases) to alter the activity of key proteins responsible for the division of plant and animal cells (see figure). Expression of B-cyclin genes in plants normally occurs only during a very brief period just before cell division^{1,3}. Doerner *et al.* inserted extra copies of the gene encoding B-cyclin into the genome of *Arabidopsis* where its expression was artificially boosted by a promoter that is active in all normally proliferating cells. They found that the number of cells in the roots was significantly increased; the roots were longer, but otherwise looked entirely normal. ▶



Are mitotic cyclins limiting for cell-cycle progression in plants? Normally transcribed during the gap (G2) between the DNA-synthesis phase (S phase) and early M phase, when the cell starts to divide, the mitotic cyclin (B-cyclin) is required to activate the mitotic Cdc2 kinase complex. This M-phase kinase drives the cell into mitosis, when the doubled-up contents of the nucleus pull apart just before the cell divides into two. The activity of this kinase is critically dependent on the availability of the B-cyclin subunit and, in some cell types, progression through the cell cycle must wait for enough cyclin to accumulate.

Experiments in which cyclins have been expressed in yeast and animal cells have not produced such striking results⁴⁻⁷, for although expression of cyclins A, D and E in fibroblasts can accelerate entry into the DNA-doubling phase of the cell cycle, there tend to be compensatory increases in the length of other phases of the cycle, so neither the rate of cell division nor cell size is dramatically affected (though what might the effects be in a whole mouse?)⁴⁻⁶. It is conceivable that cell proliferation controls, although very similar, are wired differently in plants and animals.

How might extra B-cyclin produce more cells? The *cdc2* promoter used by Doerner and colleagues should produce cyclin transcript in cells at all stages of the cell cycle. Cells that are in the G1 phase, stockpiling essential supplies before division, are probably resistant to the effects of B-type cyclins, which may not be stable in this phase of the cycle⁷, but cells that are in the later early-G2 phase that precedes nuclear division could be advanced into an early mitosis. One might expect smaller cells to result from premature mitosis, but the ultimate cell size in their transgenic plants was not reduced¹. It may be that smaller cells are transiently produced which subsequently expand to normal size during differentiation, or that cells that have moved out of the 'zone of division' are brought back into the division cycle.

Whatever the mechanism by which they arise, the fate of these extra cells is informative — the plant incorporates them into its normal body plan and carries on regardless, except faster. This is probably due to the way in which plants develop: they undergo extensive postembryonic organ formation from undifferentiated proliferative zones called meristems. As cells leave the meristematic region, divi-

sion rates decline, and cell identity is determined almost entirely by the position of one cell relative to its neighbours, rather than by its genealogy. In contrast, cell lineage plays the major role in determining the identity of animal cells. We may imagine that in the cyclin-transgenic plants, extra cells produced in the root meristem simply enlarge the pool of cells that form the root, whereas if extra cells were produced in the differentiated zone (which is unlikely, given that the *cdc2* promoter used is expressed mainly in the meristem), their identity presumably would still be fixed by their neighbours.

If increasing the number of cells produces a somewhat larger plant, then what is the effect of decreasing cell number? Hemeryly and co-workers² made transgenic plants expressing an inactive CDK protein which forms non-productive complexes with cyclins. The surprising result here is that these transgenic plants grow at almost the same rate and to almost the same size, despite containing less than half the normal number of cells. Thus, as the number of cells decreases, the size and shape of the organs are maintained by a concomitant increase in the size of individual cells. So when determining their ultimate size,

plant organs must be measuring something other than cell number alone, but what this might be remains a mystery.

This remarkable tolerance shown by plants with regard to the number of cells that they require to fabricate their body plan may one day be exploited to increase biomass (larger potatoes spring to mind). But the first step is to reach an understanding of the molecular interplay between cell-cycle controls, growth and development. □

John Doonan is in the Department of Cell Biology, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK. Tim Hunt is at the ICRF, Clare Hall Laboratories, South Mimms, Potters Bar, Hertfordshire EN6 3LD, UK.

1. Doerner, P., Jørgensen, J. E., You, R., Steppuhn, J. & Lamb, C. *Nature* **380**, 520-523 (1996).
2. Hemeryly, A. et al. *EMBO J.* **14**, 3925-3936 (1995).
3. Fobert, P. R., Coen, E. S., Murphy, G. J. P. & Doonan, J. H. *EMBO J.* **13**, 616-624 (1994).
4. Ohtsubo, M. & Roberts, J. M. *Science* **259**, 1908-1912 (1993).
5. Resnitzky, D., Hengst, L. & Reed, S. I. *Molec. cell. Biol.* **15**, 4347-4352 (1995).
6. Resnitzky, D., Gossen, M., Bujard, H. & Reed, S. I. *Molec. cell. Biol.* **14**, 1669-1679 (1994).
7. Amon, A., Imrigger, S. & Nasmyth, K. *Cell* **77**, 1037-1050 (1994).

COSMOLOGY

CAT eyes the early Universe

Michael Rowan-Robinson

In 1992 the COBE team made the headlines with the announcement that they had detected ripples in the cosmic microwave background radiation on an angular scale of 10 degrees. This radiation gives a picture of the Universe one hundred thousand years after the Big Bang, but the simplicity of cosmological models ensures that the ripples carry the imprint of much earlier epochs in the Universe, perhaps back to the hypothesized 'inflationary' era 10^{-35} seconds after the initial singularity. The modest angular resolution of COBE means that the detected ripples would be 500 megaparsecs across today, far larger than any structure we are able to identify in the observed Universe of galaxies. To make real progress in understanding the origin of galaxies and clusters of galaxies we need to study the microwave background fluctuations on much smaller angular scales.

Since the announcement of the COBE results there has been a string of confirming detections of the ripples from balloon-borne and ground-based experiments, with resolutions in the range 1 to 10 degrees. Although these results justify the splash made by the COBE discovery, there is disagreement between them, particularly at the lower end of the resolution range. Now a team of astronomers at the Mullard

Radio Astronomy Observatory in Cambridge have announced¹ the detection of microwave background fluctuations on a scale of 20 arcminutes, a factor of three better than the previous best resolution.

The team use an instrument known as CAT, the Cosmic Anisotropy Telescope, which is a three-element interferometer operating at frequencies between 13 and 17 GHz, at Lords Bridge outside Cambridge. Using 400 hours of data from CAT, rejecting 40 per cent of the data as excessively noisy because of bad weather and carefully subtracting foreground astronomical sources, they have made a detailed map of the microwave sky over a square about 2 degrees across. On scales between 14 and 27 arcminutes the amplitude of the r.m.s. temperature fluctuations in the microwave background is $55 \pm 11 \mu\text{K}$, compared with the observed temperature of the background of 2.73 K. The amplitude seen by COBE on large scales was about 20 μK .

What conclusion can we draw from these new results? The form of the fluctuation spectrum as a function of scale is a very sensitive measure both of cosmological parameters and of the nature of the underlying density fluctuations. Models of the Universe dominated by cold dark matter predict that there should be a

dominant peak in a plot of fluctuation amplitude against angular scale, at a scale that ranges from 1 degree for a Universe of critical density to 15 arcminutes for a Universe with one-tenth of the critical density, as well as other, smaller amplitude peaks on smaller scales. These 'Doppler' peaks arise because the coupled fluid of luminous matter and photons undergoes acoustic oscillations just before the recombination epoch where the microwave background is produced. The scale of the dominant peak is related to the size of the 'horizon'—the limiting distance at which different parts of the Universe have had time to interact with each other—at the epoch at which we are viewing the radiation.

Some recent reviews² claim that the observations on scales of 1 to 10 degrees support the existence of a peak at 1 to 2 degrees, consistent with a cold dark matter Universe of critical density. Scott *et al.* suggest that the CAT observations indicate a decline in amplitude towards smaller scales, which is inconsistent with models assuming a low-density Universe. If the first Doppler peak could be firmly placed on a scale of 1 to 2 degrees, this would also be inconsistent with the predictions³ of a completely different mechanism for the origin of structure—postulated objects called cosmic strings, one-dimensional topological defects left over from a phase transition in the early Universe.

To the unprejudiced eye, however, the current observations are perfectly consistent with a steady increase in fluctuation amplitude from 10 degrees down to 20 arcminutes, so low-density Universes and string models cannot yet be ruled out. The accuracy of the measurements on scales of 1 to 10 degrees needs to improve substantially before we can reach such strong conclusions. But these results demonstrate the great potential of accurate measurements of microwave background fluctuations on small scales. Future exciting developments include forthcoming results from the Princeton group's Saskatoon experiment on scales of 1 to 10 degrees, the Cambridge group's proposed Very Small Array which will push these studies to even smaller scales and the COBRAS/SAMBA space mission proposed for launch by the European Space Agency in 2004, on which a decision will be made next month. This has the potential to determine the main cosmological parameters to an unprecedented accuracy of 1 per cent, and move cosmology into a new era of precision. □

Michael Rowan-Robinson is in the Department of Physics, Blackett Laboratory, Imperial College, London SW7 2BZ, UK.

A two-way structure

R. S. Goody

MOTOR proteins are among the most interesting objects of study in the biological sciences: they are involved in a wide range of processes essential to life, including intracellular organelle movement, cell division, cell motility and muscle contraction. The three-dimensional structure of the motor unit of the classical representative of this group, muscle myosin (myosin II), was determined several years ago¹, and similar information on a representative of another important class of molecules involved in intracellular motility has been awaited eagerly.

On pages 550 and 555 of this issue, Kull *et al.*² and Sablin *et al.*³ report crystal structures of the motor domains of two such proteins, kinesin and *ncd*, which are closely related. These exciting structures suggest that the mechanism of energy transduction in these two proteins and myosin is similar at the level of the active sites, but that different strategies may be used to amplify small local conformational changes into the larger movements needed at each round of ATP hydrolysis.

Like myosin II, kinesin and related molecules are double headed owing to the presence of two identical dimerized heavy chains. But the motor unit of kinesin (some 340 amino acids in length) is only a little more than one-third of the size of myosin's, so it is no surprise that there is, at first sight, no obvious similarity in the gross morphology of the myosin and kinesin heads.

The surprise comes on detailed comparison of the structures. This is achieved by aligning the P-loop, which is involved in binding the phosphate groups and is conserved in a large number of ATP- and GTP-utilizing enzymes, including the ATP-driven motor proteins, GTPases of the heterotrimeric type and of the Ras family, nucleotide kinases and RecA. The comparison revealed a high degree of overlap of secondary structure elements between myosin, kinesin and *ncd* (which is structurally very similar to kinesin), but not between these and the other P-loop proteins. Thus, kinesin fits snugly into the myosin head, with spatial overlap of the six major helices and seven of the eight core β -strands of kinesin. Although there are some differences in the topological connections between these elements, this similarity could indicate that both types of molecule have evolved from a common precursor.

The main differences between the myosin and kinesin structures are in the size and nature of the loops and larger insertions between these conserved secondary structural elements. Kull *et al.* and Sablin *et al.* propose that it is these differ-

ences which determine, among other things, the known differences in ATPase kinetics of the two types of motor. Such a role (limiting the rate of ADP release) was suggested earlier for a loop near the active centre of myosin⁴.

The state of the nucleotide at the active site of a motor protein must be linked to two properties of the contractile system—namely, to the affinity for the corresponding polymeric partner protein (actin for myosin, microtubules for kinesin and dynein), and to movement and force generation. These two features could be closely linked in space, placing the point about which a conformational change takes place near or at the interface between the motor and polymeric proteins. But more recent thinking, stimulated by the myosin structures, places the pivot at the carboxy-terminal end of the ATPase domain of the myosin head, which is distal to the actin-binding site and at the start of an extended helix which is stabilized by interaction with light chains.

In a currently discussed model, relatively small structural changes at the active site of myosin resulting from ATP hydrolysis are amplified by a rotation of this lever arm, and there is electron microscopic evidence that a change of angle of the lever arm of at least 30° can indeed occur⁵. In the kinesin and *ncd* head structures, such a structural element is not present, but there is evidence from other studies that the next 40 or so amino acids in the kinesin structure can form a helix and a coiled-coil with the second molecule in the dimer. Thus, the amino-terminal 340-amino-acid fragment of kinesin is monomeric, whereas longer constructs are dimeric. However, assuming a similar angle change to that seen in myosin, the length of this coiled-coil neck is too short to explain the length of discrete steps in the kinesin contraction mechanism (about 80 Å; ref. 6).

Kull *et al.* and Sablin *et al.* therefore favour a model in which a large change in the angle of a lever arm is not the basis of movement, but that a conformational change in one head biases the direction in which the other head will seek the next tubulin-binding site 80 Å away. This type of hand-over-hand model has been discussed previously⁷ and evidence for half site reactivity in dimeric kinesin fragments has been presented⁸. This would explain the observation of processive movement of dimeric (but not monomeric) kinesin along microtubules without dissociation⁹, because one head would always be in a strongly bound state.

The ideas discussed in the papers and here could mean that, despite similarities of mechanism at the active-site level, differ-

1. Scott, P. F. *et al.* *Astrophys. J.* **461**, L1–L4 (1996).
2. White, M., Scott, D. & Silk, J. A. *Rev. Astr. Astrophys.* **32**, 319 (1994).
3. Maguiejo, J., Albrecht, A., Coulson, D. & Ferreira, P. *Phys. Rev. Lett.* (submitted).

ent techniques for the amplification of small structural changes near the active site are used in the myosin and kinesin cycles. It is noticeable that kinesin's two-headedness plays an important role at different levels of observation (ATPase kinetics, movement), whereas the significance of myosin's two-headedness remains obscure. Differences between monomeric and dimeric myosin fragments seem to be minor and quantitative rather than qualitative, as for kinesin. The lever arm in the myosin molecule is a feature of each of the two heads, whereas the neck region of kinesin is a single structure shared by both heads.

As Sablin *et al.* suggest, the common basic mechanism may also extend to other proteins, because although alignment of the P-loop of GTPases with that in kinesin does not lead to overlap of the secondary structure elements, it does result in a remarkable alignment of the nucleotides and several conserved sequence elements. In particular, there are obvious counterparts to the switch I and switch II regions of the GTPases. These are regions known to undergo major structural changes on GTP hydrolysis, and are the primary elements involved in sensing the γ -phosphate group^{10,11}.

What must still be defined is the manner in which such changes are converted into structural changes resulting in motion. That very subtle effects are involved in this process is illustrated dramatically by the fact that the structurally highly similar motor domains of kinesin and ncd promote movement in opposite directions along microtubules — a result that is also more easily reconciled with the hand-over-hand mechanism than with a mechanism in which a large angle change of bound heads occurs. Whether the opposite polarity of movement is related to the finding that molecules of this family which have the motor unit at their amino terminus (like kinesin) move in one direction, whereas those having the motor unit at their carboxy terminus (like ncd) move in the other, is a fascinating question. It remains to be answered, and might be testable with engineered chimaeric proteins. □

R. S. Goody is in the Department of Physical Biochemistry, Max-Planck-Institut für Molekulare Physiologie, Rheinlanddamm 201, 44139 Dortmund, Germany.

1. Rayment, I. *et al.* *Science* **261**, 50–58 (1993).
2. Kull, F. J., Sablin, E. P., Lau, R., Fletterick, R. J. & Vale, R. D. *Nature* **380**, 550–555 (1996).
3. Sablin, E. P., Kull, F. J., Cooke, R., Vale, R. D. & Fletterick, R. J. *Nature* **380**, 555–559 (1996).
4. Spudis, J. A. *Nature* **372**, 515–518 (1994).
5. Jontes, J. D., Wilson-Kubalek, E. M. & Milligan, R. A. *Nature* **378**, 751–753 (1995).
6. Svoboda, K., Schmidt, C. F., Schnapp, B. J. & Block, S. M. *Nature* **365**, 721–727 (1993).
7. Peskin, C. S. & Oster, G. *Biophys. J.* **68**, 202s–211s (1995).
8. Hackney, D. D. *Proc. natn. Acad. Sci. U.S.A.* **91**, 6865–6869 (1994).
9. Vale, R. D. *et al.* *Nature* **380**, 451–453 (1996).
10. Milburn, M. V. *et al.* *Science* **247**, 939–945 (1990).
11. Schlichting, I. *et al.* *Nature* **345**, 309–315 (1990).

DNA TYPING

Heteroplasmy and the Tsar

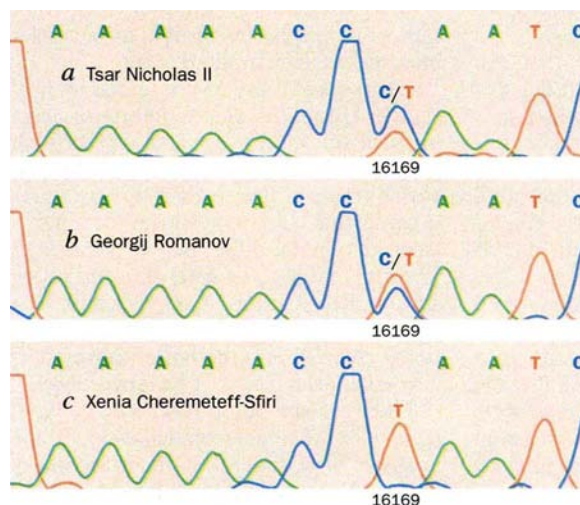
Paul G. Debenham

THE trick in making a great sequel is to pick up the cliffhanger ending from the first story and deliver the punchline, while revealing a hidden plot, or unexpected perspective, that lay beneath the surface of the original picture. Two years ago Pavel Ivanov, along with Peter Gill and associated forensic and anthropology stars, presented an epic tracing the identification of Tsar Nicholas II's bones exhumed from a grave in Ekaterinburg in

the individuals' remains from Ekaterinburg were consistent with their being those of the Romanov family. But the proof of maternal inheritance, which should have linked the putative Tsar's bones to the known living relatives of the Tsar's family, ended up with the ambiguous finding of genetic heteroplasmy between the bones and the descendants.

Part *a* in the figure shows a snapshot of the DNA sequence of the hypervariable region of mitochondrial DNA obtained from the Tsar's bones. At base position 16169, the DNA sequence reveals the presence of both a cytosine (C) and a thymine (T). This result was repeatedly found and cannot be attributed to contamination or artefact. Thus the Tsar presumably must have had two populations of mitochondria within his cells that contained either a C or a T at this position.

This heteroplasmy confounded the confidence of proving a link (mitochondria are maternally inherited) to the great granddaughter of Tsar Nicholas II's sister. Countess Xenia Cheremeteff-Sfiri is that descendant and her mitochondrial sequences match with the exception of position 16169 where only a T is found (part *c* in the figure). So the first study ended with the implication that the remains were those of the Tsar if only the mitochondrial sequencing discrepancy could be resolved; thus the



Romanov lines — these automated sequence chromatographs show the mitochondrial DNA sequences at the crucial 16169 position of the presumed Tsar Nicholas II, his brother (Georgij Romanov, the Grand Duke of Russia) and a great granddaughter of the Tsar's sister (Countess Xenia Cheremeteff-Sfiri). At position 16169, the Tsar's mitochondrial DNA can be either cytosine (C) or thymine (T), the former predominating. Such a mixture of bases at the same position is known as heteroplasmy. Two living relatives from the same matrilineage — Xenia Cheremeteff-Sfiri and the Duke of Fife (sequence not shown) — are homoplasmic for thymine at 16169. But the new results show that, like the Tsar, his brother is heteroplasmic at that position, this time with T predominating over C. The results confirm that the remains concerned are those of the Tsar; and the swift intergenerational switch from heteroplasmy to homoplasmy presents geneticists with a puzzling scientific issue.

Russia¹. Despite calling on the ultimate arbiter, DNA sequencing, the analyses left a chink of doubt as to whether the remains were truly those of the Tsar.

In this month's *Nature Genetics*², Ivanov and a new cast of investigators now deliver clinching evidence that the bones are indeed the Tsar's. At the same time they have unearthed an intriguing aspect of genetic inheritance that was the unexpected cause of the unresolved crisis of identity in part one of the story.

The figure reproduced here, which comes from the new paper², encapsulates both the original part of the story and its sequel. Genomic DNA typing using short tandem repeat loci, as well as skeletal and dental records, had indicated that

plot was nicely set up with an uncomfortable ending begging for the sequel.

The Russian authorities did not rest with this ending and asked Ivanov to analyse the recently exhumed remains of the Grand Duke of Russia Georgij Romanov, who was the brother of Tsar Nicholas II. Now at last sequencing delivers the goods and reveals the sub-plot. Lane *b* in the figure provides the missing link. The Tsar's brother has a matching hypervariable sequence with the same heteroplasmy at position 16169. Irrespective of the statistical estimates calculating the significance of this match, the sequence data are compelling and will convince all but the most dogmatic of sceptics that the bones have been correctly identified. ▶

The sub-plot that mitochondrial sequences can evolve rapidly is just as exciting. Although the two brothers share the same heteroplasmy, they presumably inherited from their mother very different proportions of the C and T at position 16169. Further, the type T mitochondria must have segregated to homoplasmy within the four generations from the Tsar's mother to Xenia. This royal documentation of rapid mitochondrial evolution in humans will prompt some serious thinking about the mechanisms of mitochondrial selection and transmission. But for those of us in the audience watching the progress of human genetics it also

perhaps indicates that we may have too simplistic a view of the inheritance of human characteristics as being solely down to the genetics of the nucleus. The subcellular population drifts of mitochondrial genetics through the generations may yet have a surprise or two in store for those beavering away on the Human Genome Project. □

Paul G. Debenham is at University Diagnostics Ltd, South Bank Technopark, 90 London Road, London SE1 6LN, UK.

1. Gill, P. et al. *Nature Genet.* **6**, 130–135 (1994).
2. Ivanov, P. L. et al. *Nature Genet.* **12**, 417–420 (1996).

NEUROPSYCHOLOGY

The brain's dictionary

Alfonso Caramazza

How is knowledge of words organized in the brain? Hanna Damasio and her collaborators (page 499 of this issue¹) may have provided part of the answer to this complex question. They report evidence that suggests that lexical knowledge is organized by category in distinct areas of the left temporal lobe. Their proposal distinguishes among three neural systems: one represents the conceptual content (the meaning) of words; another represents the phonological elements (sounds) that compose words; and the third, mediating between the first two, represents modality-independent lexical knowledge. It is this third system that Damasio and her collaborators have localized to the left temporal lobe.

This proposal contains the basic claims that there is a level of lexical representation that mediates between conceptual and phonological knowledge of words; that knowledge of words is organized cat-

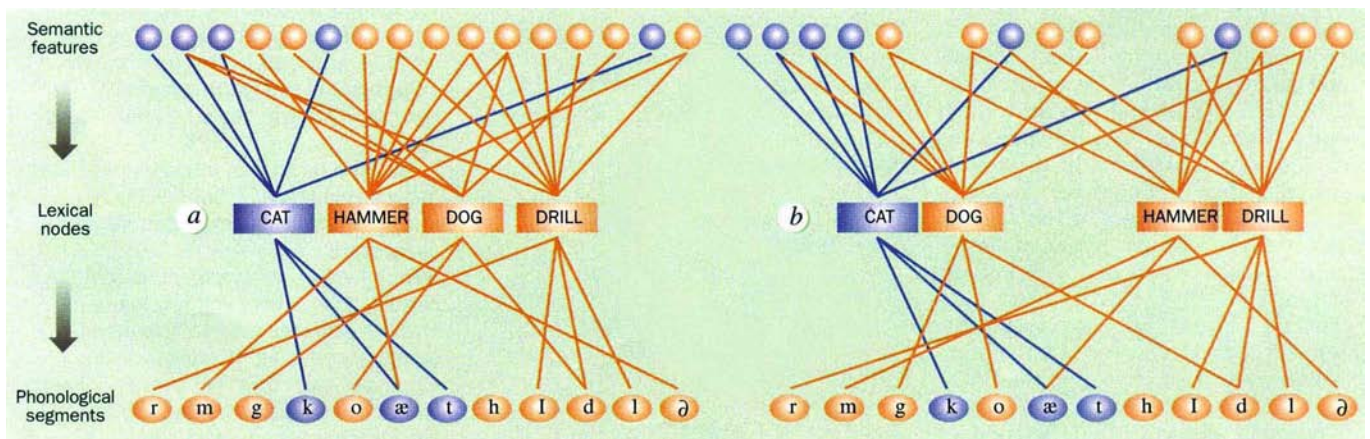
egorically; and that the part of word knowledge that is organized categorically in the left temporal lobe is the lexical level and not the conceptual or phonological levels. The first two claims are widely accepted and are supported by various sorts of experimental evidence. The last claim is new, and is the subject of Damasio and colleagues' study.

Cognitive scientists have long maintained that, in speech production, word retrieval involves two stages of processing: representations at the conceptual level are initially used to select modality-independent lexical representations; these lexical representations are then used to select specific phonological forms. In other words, it is thought that there are abstract lexical representations mediating between conceptual knowledge and the phonological form of words (*a* in the figure). These representations are said to be abstract or modality-indepen-

dent because they specify the words of the language without directly providing information about their phonological or orthographic forms. There is compelling experimental evidence in support of this hypothesis^{2,3}.

Similarly compelling is the evidence that semantic categories (that is, categories such as furniture, birds, fruits and tools) play a crucial role in the organization of word knowledge in the brain. The neuropsychological literature contains sporadic references to the existence of category-specific deficits at least since 1936, when Nielsen⁴ described one patient who had much more difficulty in naming living than non-living things, and another who had the reverse problem. But it was not until Warrington's⁵ seminal work on semantic memory, published in 1975, that such deficits were explored in enough detail to permit firm conclusions about their significance for theories of the organization of word knowledge in the brain. Since then, a large number of detailed reports have conclusively demonstrated that brain damage, for instance as the result of a stroke, can result in highly selective deficits of semantic memory involving specific conceptual categories. For example, there have been many reports of selective impairment⁶ or 'sparing'⁷ in use of words in the category 'animals'. Other semantic category-specific deficits (for example for tools, proper names, fruits and vegetables, and so on) have also been described⁷. Furthermore, it has been shown that the category effects are not due to extraneous factors such as the relative familiarity or complexity of one or another semantic category.

Nevertheless, the precise interpretation of these semantic category deficits remains unresolved. One interpretation assumes that they reflect either damage to semantic properties shared by members of



a, The three levels of representation of word knowledge necessary for speech production. Semantic features (carnivorous, furry, domesticated, pet) activate lexical nodes (the word CAT) which, in turn, activate their corresponding phonological features (k, æ, t). *b*, Organization of the three levels in the form proposed by Damasio et al.¹. The neural system for conceptual information consists of a distributed network

involving structures in both the left and right hemispheres. These networks are connected to lexical representations in the left temporal lobe which are organized by semantic category — animals in the inferior temporal (IT) lobe; tools in the posterior regions of the IT lobe and the occipito-temporo-parietal junction. The selected lexical representation in turn activates its associated phonological features for speech production.

a particular category or damage to the pathways leading from those semantic properties to lexical representations^{6,7}. Damasio *et al.*¹ propose, instead, that category-specific deficits may reflect the categorial organization of the mediating lexical representations (*b* in the figure). They base their conclusion on the results of two studies — one involving anatomical correlations, the other involving functional imaging with positron emission tomography (PET).

The principal evidence they provide is the correlation between category-specific naming deficits and sites of brain damage. To maintain that the category effects in naming disorders reflect specifically the organization of lexical (and not conceptual) representations, it must be shown that naming failures do not result from damage to the semantic system. To this end, a naming trial was scored as an error only when patients demonstrated comprehension of the object by providing an adequate description but still failed to produce the correct name (for instance when they could describe a skunk as a small, black-and-white animal that makes a nasty smell, but not actually name it). In this way, Damasio *et al.* argue, we may be sure that category-specific naming failures can be attributed to a deficit in lexical retrieval and not in semantic processing.

Using this procedure, they found that patients who were selectively impaired in producing people's names had damage restricted to the left temporal pole (TP); patients who were selectively impaired in producing animal words had damage restricted to the left inferior temporal (IT) lobe; and patients who were selectively impaired in producing the names of tools had damage to the posterior inferior temporal lobe and the temporo-occipitoparietal junction (posterior IT+) (see Fig. 2 of the paper on page 501).

Converging evidence for this conclusion came from a parallel PET study with neurologically intact individuals: significantly greater activation was found in the left TP, IT and posterior IT+ in naming people, animals and tools, respectively. The close correspondence in the results obtained in the PET and the anatomical studies provides a compelling basis for the conclusion that word knowledge is organized categorially in the left temporal lobe.

There is a pleasing elegance to the proposed theoretical framework for the organization of word knowledge in the brain. The autonomous level of lexical representation serves the important function of providing focal points for collecting the set of distributed conceptual and phonological features that constitute, respectively, the meaning and the pronunciation of words. The localization of these lexical representations to specific areas of the left

temporal lobe is an important achievement. The proposal that these lexical representations are organized by semantic category is equally important, although it is not uncontroversial because other imaging studies have found maximal activation for the animal and tool categories in other areas of the left hemisphere⁸. We also cannot exclude the possibility that the category-specific deficits in Damasio and colleagues' patients involved semantic rather than lexical representations.

Further studies will be needed to resolve these potential difficulties, and to address aspects of lexical representation not tackled by Damasio *et al.* Their study was restricted to concrete concepts. Are abstract concepts — justice, evidence and ambition, for example — also represented categorially? Are they, too, represented in the temporal lobe? And what about syntactic information? Lexical representations must also serve as the focal points about the grammatical class of words and their selectional restrictions (for instance that the verb 'give' but not 'die' requires a direct object)^{2,3}. It is obvious the syntactic features of words must be represented in the brain, but not how this information is organized in relation to other aspects of word knowledge.

What is clear, however, is that brain damage can result in selective deficits for individual grammatical classes of words. For example, patients have been reported with selective deficit⁹ or sparing¹⁰ in use of verbs, nouns or function words (articles, auxiliaries and prepositions). These results, like those for semantic categories, suggest some form of categorial organization at the lexical level of representation. It remains to be determined how the categorial structure for syntactic features is related to that for semantic categories, but the combined approach of anatomical and functional imaging studies provides a promising way forward. □

Alfonso Caramazza is in the Cognitive Neuropsychology Laboratory, Department of Psychology, Harvard University, 33 Kirkland Street, Cambridge, Massachusetts 02138, USA.

caram@wjh.harvard.edu

1. Damasio, H., Grabowski, T. J., Tranel, D., Hichwa, R. D. & Damasio, A. R. *Nature* **380**, 499–505 (1996).
2. Garrett, M. *Cognition* **42**, 143–180 (1992).
3. Levelt, W. J. M. *Speaking: From Intention to Articulation* (MIT Press, Cambridge, MA, 1989).
4. Nielsen, J. M. *Agnosia, Apraxia, and Aphasia: Their Value in Cerebral Localization*, 2nd edn (Paul B. Hoeber, New York, 1946).
5. Warrington, E. K. Q. *Jl exp. Psychol.* **27**, 635–657 (1975).
6. Warrington, E. K. & Shallice, T. *Brain* **107**, 829–853 (1984).
7. McCarthy, R. & Warrington, E. K. *Proc. R. Soc. B* **346**, 89–96 (1994).
8. Martin, A., Wiggs, C. L., Ungerleider, L. G. & Haxby, J. V. *Nature* **379**, 649–652 (1996).
9. Caramazza, A. & Hillis, A. E. *Nature* **349**, 788–790 (1991).
10. De Renzi, E. & di Pellegrino, G. *Cortex* **31**, 619–636 (1996).

DAEDALUS

Variable voltage

THE transformer, that elegant invention which steps some voltage up or down in almost every electrical or electronic system in the world, has one major drawback. It only works with alternating current — indeed, that is why all power distribution systems use a.c. Daedalus is now inventing a true d.c. transformer.

He points out that a current flows in a wire because each electron is pushed along by the repulsion of the electrons behind it. They need not be exactly behind it; even an electron behind and to one side will exert a component of its repulsion in the direction of the current. Even an electron so far to one side that it is in an adjacent wire will exert some repulsion. So, says Daedalus, a steady current in one wire should create one in another wire alongside, simply by this diagonal repulsion. This is a d.c. transformer.

Sadly, electron repulsion falls off rapidly with distance. For an electron in one wire to influence one in another, the wires would need to be almost atomically thin and adjacent. Modern monolayer-film and vapour-deposition techniques can lay down such layers quite easily. So Daedalus's d.c. transformer consists of many alternate layers of metal and insulator, each only a few atoms thick, interleaved so that a current in the input layers induces a current in the output layers between them. If each input layer is a single wide strip, while each output layer is many narrow strips side by side and connected in series, the result will be a step-up transformer. Connected in reverse, it will step a voltage down.

In step-up form, the d.c. transformer will be a wonderful saver of batteries. All batteries lose voltage as they run down. We usually discard them long before they are exhausted, simply because their voltage has dropped too low. But an adjustable d.c. transformer in the circuit could be arranged to step up the voltage automatically as fast as it declined. The last drop of power could be wrung from every battery.

The step-down d.c. transformer will make possible a new battery. Many radioactive sources emit electrons or alpha-particles of many millions of electron volts — a tiny beam of 'primary current' at very high voltage. Fired at grazing incidence past an appropriate secondary winding, it could induce a much stronger current at a usefully lower voltage. Radioactive waste, now a pure liability, could power neat and highly efficient little 'radio-batteries' that might last for decades. They would be ideal power sources for isolated areas, or unmanned relay or scientific stations, or even spacecraft.

David Jones

Risks from transgenic crops

SIR — Debate continues over the possible risks posed by transgene movement via pollen following the commercial release of genetically modified oilseed rape^{1,2}. We have investigated the likelihood of glufosinate-tolerant transgenes first moving into and then influencing the survival of feral populations of the crop.

We monitored airborne pollen densities at 0 m, 100 m, 360 m and 1.5–2.5 km from isolated oilseed rape fields (3–10 ha) for three years. Densities at 360 m were 10–12% of those at the field margin; we consistently observed low densities at 1.5–2.5 km. It has been reported³ that the decay in pollen concentration, C , with distance, r , from a source could be approximated by an exponential function $Cae^{-r/\alpha}$, where α is the characteristic dispersal distance. The value of α for oilseed rape has been calculated³ to lie between 2.3 and 8.8 m. The same function fitted to our data, however, suggests that α lies between 128 and 172 m, with 95% confidence. The use of an exponential rather than a power-law model⁴, and the subtraction of pollen levels at 2 km as a background correction,

makes this a conservative estimate of long-range dispersal. We suggest the increased value of α presented here can be explained at least partly by our use of commercial-sized fields, 24-hour monitoring and widely spaced sampling sites. Our results show that significant quantities of pollen travel over large distances; this has implications for transgene recruitment by feral populations, provided pollen viability and competitiveness are unaffected by dispersal.

The proportion of potential seeds on stands of emasculated plants showed the same dependence on distance from an isolated field as the pollen profile, suggesting that pollen viability is unaffected by dispersal. We also exposed emasculated and subsequently self-pollinated plants (cv. Comet) to airborne pollen from an isolated field (cv. Libravo). We identified intercultural hybrids at 0 m (6.3%, 8/126), 100 m (0.5%, 1/195) and 360 m (3.7%, 5/135) by simple-sequence repeat PCR (polymerase chain reaction) and RAPD (randomly amplified polymorphic DNA) analyses (Fig. 1). The significance of these results for the levels of gene flow expected

in the natural environment depends on the proximity of fields and feral populations. In our three-year survey (1993–95) of a 480-km² area of Angus, Scotland, for example, we found that between 31% (28/89) and 42% (35/83) of feral populations were sited within 360 m of an oilseed rape field.

It follows that attention should now focus on the probable effects of any introduced transgenes. In the case of a glufosinate-tolerant cultivar, it is important to establish whether the transgene would confer direct selective advantage in the feral environment, as might be expected if the herbicide is widely applied (Fig. 2). Our survey of feral populations in 1995 revealed that 44% had been subjected to control measures. The most widely used method, mowing, was applied to 39% of populations. Herbicides were used on only 5.7% of populations; we found no evidence of glufosinate application using any of the commercial formulations available. Close examination of five populations sprayed with herbicides revealed that all contained survivors which set seed. Thus, although tolerant individuals would have clear selective advantage if sprayed, our results suggest that few populations are likely to receive such treatment, and that even in these, not all plants would be affected.

We infer that, in the area surveyed, the possession of glufosinate-tolerance is unlikely to affect the survival of feral populations significantly. This assertion takes no account of future changes in glufosinate application in non-agricultural situations, of pleiotropic effects of the transgene, or of effects on agricultural volunteers. Our work demonstrates the need for a careful, case-by-case approach to the risk assessment of genetically modified organisms.

A. M. Timmons*

Y. M. Charters

J. W. Crawford†

D. Burn

S. E. Scott*

S. J. Dubbels

N. J. Wilson

A. Robertson

E. T. O'Brien

G. R. Squire

M. J. Wilkinson*

Scottish Crop Research Institute,
Invergowrie, Dundee DD2 5DA, UK

1. Coghlan, A. *New Scientist* **145** (1967), 5 (1995).
2. Mikkelsen, T. R., Andersen, B. & Jorgensen, R. B. *Nature* **380**, 31 (1996).
3. McCartney, H. A. & Lacey, M. E. *J. agric. Sci., Camb.* **107**, 299–305 (1991).
4. Shaw, M. W. *Proc. R. Soc. Lond.* **259**, 243–248 (1995).
5. Charters, Y. M., Robertson, A., Wilkinson, M. J. & Ramsay, G. *Theor. appl. Genet.* (in the press).

*Present addresses: Plant Science Department, Scottish Agricultural College, Auchincruive, Ayr KA6 5HW, UK (A.M.T.); Department of Agricultural Botany, Plant Science Laboratories, The University of Reading, Whiteknights, PO Box 221, Reading RG6 6AS, UK (S.E.S., M.J.W.).

† To whom correspondence should be addressed.

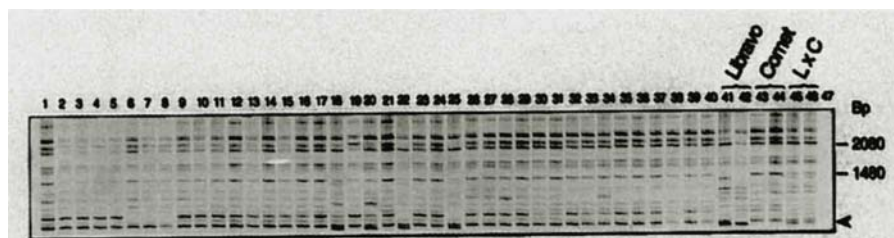
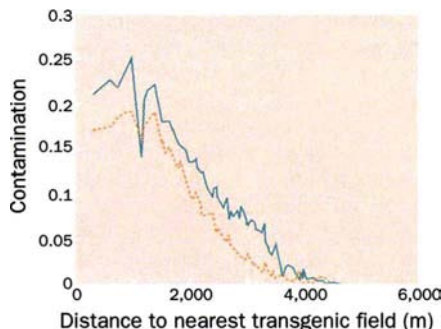


FIG. 1 Identification of hybrids between cultivars Libravo and Comet using the 5' anchored simple-sequence repeat primer 1425 and PCR according to ref. 5. Lanes 1–40, offspring of emasculated plants (cv. Comet) sited 360 m from the field (cv. Libravo); lanes 41, 42, cv. Libravo; lanes 43, 44, cv. Comet; lanes 44–46, F₁ hybrids (cv. Libravo × cv. Comet); lane 47, control. Intercultural hybrids can be identified in lanes 18, 20, 22 and 25 by the presence of a Libravo-specific fragment (arrowed). Hybrid status was checked using a 900-base-pair Libravo-specific fragment obtained after RAPD analysis using primer 5'-CCACCGCCAG-3'. Band homology was confirmed by Southern analysis.

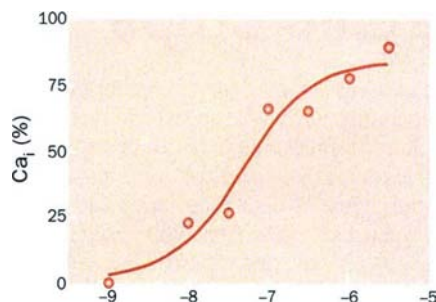
FIG. 2 Simulated average proportion of transgenic seed set in wild-type feral populations as a function of distance from the nearest genetically modified field in a region where 10% of oilseed rape fields are transgenic. Feral population sizes were assumed to follow the frequency distribution observed in the survey. The proportion of genetically modified to non-genetically modified seed set in a feral population was assumed to be proportional to the relative concentration of airborne pollen originating from each type of pollen source. The curves result from a numerical solution of the equation $\delta C/\delta t = D \nabla^2 C - v \nabla C - kC$, where C is airborne pollen concentration, D is the dispersal coefficient, v is the prevailing wind velocity and k is a first-order deposition constant. Values of k and D are derived from our pollen-trap data. Solid line, the case where fields are less clustered (fractal dimension of 1.26); dotted line, fields which are more clustered (dimension of 1). The number of feral sites, non-genetically modified and genetically modified fields is identical in both cases, and each point represents the average of three (more clustered) or five (less clustered) random realizations of the spatial distribution of field and feral sites.



Neural control of dieting

SIR — Tecott *et al.*¹ have shown that transgenic mice lacking the receptor for the 5-hydroxytryptamine neurotransmitter subtype 5-HT_{2C} become obese through overeating, indicating a primary role for this receptor in the satiety response; these animals do not respond to the 5-HT_{2C} receptor agonist *m*-chlorophenylpiperazine (mCPP). Cowen *et al.*² emphasized that antagonists for this receptor, such as clozapine and mianserin, cause troublesome weight-gain in people; clozapine abolishes the endocrine responses to mCPP in humans³. These authors showed that dieting may be difficult because of an imbalance of brain 5-HT release and 5-HT_{2C} receptors: dieting lowers plasma tryptophan, the precursor for brain 5-HT, which lowers levels of this neurotransmitter in the brain, but secondarily increases postsynaptic 5-HT_{2C} receptor sensitivity². Cowen *et al.* showed an increase in 5-HT_{2C} receptor sensitivity by administering mCPP to women undergoing a 1,000-kcal daily diet; they observed a marked increase in the prolactin response among those who dieted, and suggested that lowered 5-HT levels cause receptor supersensitivity.

Dexfenfluramine is a widely prescribed dieting agent; it has been shown to be effective in causing weight loss⁴, although the mechanism has not been fully defined. Dexfenfluramine causes a marked release of 5-HT from neurons by inhibiting uptake of this neurotransmitter and by direct release⁵. In this way, the drug will counteract the effects of reduced tryptophan levels. However, dexfenfluramine has negligible affinity for 5-HT receptors. The principal metabolite of dexfenfluramine, (+)norfenfluramine, is a potent ligand in displacing [³H]-mesulergine binding to human recombinant 5-HT_{2C} receptors transfected into cultured CHO cells, with a K_i of $1.6 \pm 0.3 \mu\text{M}$ (n_{Hill} , 0.8 ± 0.1 ; dexfenfluramine K_i , $16.4 \pm 3.3 \mu\text{M}$; n_{Hill} , 1.0 ± 0.2), confirming previous binding experiments in neuronal tissue⁵. Brain levels of (+)norfenfluramine in rats given dexfenfluramine at an appropriate anorectic dose (1.3 mg per kg intraperitoneally) are of the order of 3.6 nmol per g (ref. 6), several times the threshold for receptor occupation. We also show that (+)norfenfluramine is an agonist at 5-HT_{2C} receptors, as (+)norfenfluramine



Cytosolic calcium levels in CHO cells transfected with the human recombinant 5-HT_{2C} receptor. The concentration-response curve for (+)norfenfluramine for increase in intracellular calcium is expressed as a percentage of the final response to 5-HT (3 μM).

(0.1–10 μM) increases calcium levels in the transfected CHO cells (see figure); this effect is blocked by the mixed 5-HT_{2A/5-HT_{2C}} receptor antagonist mesulergine (100 nM). The 5-HT_{2C} agonist (-)-2,5-dimethoxy-4-iodoamphetamine (3 μM) mimics the effects of (+)norfenfluramine by increasing intracellular calcium in CHO cells transfected with the 5-HT_{2C} receptor. Thus, dexfenfluramine shows a unique pharmacological spectrum by releasing brain 5-HT, which will also prevent 5-HT_{2C} receptor

supersensitivity, and also by acting, via its principal metabolite (+)norfenfluramine, as an agonist at 5-HT_{2C} receptors and thereby directly inducing satiety. The effects of dexfenfluramine as an anorectic agent in humans are abolished by the co-administration of ritanserin⁷.

These findings emphasize the role of 5-HT_{2C} receptors in controlling food intake and satiety, and suggest that dexfenfluramine might reset the balance between 5-HT release and 5-HT_{2C} receptor stimulation. Further work is required to define how control of this receptor system parallels other, recently described, systems such as glucagon-like peptide-1 (ref. 8) and leptin^{9,10}, to modulate satiety and obesity. In this respect, both the leptin receptor and 5-HT_{2C} receptors are highly expressed in the choroid plexus and, to a lesser extent, the hypothalamus.

M. Spedding

C. Ouvry

M. Millan

J. Duhalet

C. Dacquet

*Institute de Recherche Servier,
125 Chemin de Ronde,
Croissy sur Seine 78290, France*

R. Wurtman

*Department of Brain and
Cognitive Sciences,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139, USA*

Counting species names

SIR — Many estimates of global biodiversity depend critically on the extent to which species have been catalogued^{1–3}. Estimating the number of recorded species is not straightforward, because an unknown fraction of published descriptions refers to previously named species^{1,4}. It has recently been proposed that the overall fraction of synonyms can be estimated from extrapolations of revisions of particular groups⁵. However, the statistics involved are tricky, and it is unclear whether the groups used to estimate 'synonymy rates' are representative¹.

The point can be illustrated with three groups of molluscs from the Mediterranean region. Melanopsids are relatively large, conspicuous freshwater snails. In this century, the number of species accepted by different workers has progressively diminished from more than 100 to only one, extremely variable, exceedingly ancient 'species'⁵. Thus, the estimated synonymy rate in this group ranges from 0 to more than 99%. Comparative anatomy and molecular genetics unequivocally show that the number of species is indeed fairly large⁶. A taxonomic revision in progress indicates that the amount of synonyms is probably near 40%.

Freshwater mussels (unionoids) have a similar taxonomic history. Many species

were described using criteria that few, if any, contemporary biologists would agree upon. These names were progressively lumped into only seven quite variable 'species' (excluding those living in the Nile), resulting in huge lists of synonyms. However, there is now strong and growing evidence that this group has undergone a remarkable diversification in the area⁷; even so, this upwards revision has only a slight impact on the synonymy rate, which drops from 99 to 93%.

A very different situation occurs in hydrobiids, minute snails often living in springs and subterranean waters. Their small size and the general absence of conspicuous characters on their shells have kept them away from taxonomists. But most of the 50 species described in Iberia⁸ represent valid species, and indeed there

1. Tecott, L. H. *et al.* *Nature* **374**, 542–546 (1995).
2. Cowen, P. J., Clifford, E. M., Williams, C., Walsh, A. E. S. & Fairburn, C. G. *Nature* **376**, 557 (1995).
3. Kahn, R. S. *Biol. Psychiat.* **35**, 903–908 (1994).
4. Guy-Grand, B. *et al.* *Lancet* **II**, 1142–1145 (1989).
5. Caccia, S. *et al.* *Naunyn-Schmiedeberg's Arch. Pharmac.* **347**, 306–312 (1993).
6. Garattini, S. *Int. J. Obs.* **16**, S41–S48 (1992).
7. Goodall, E. M. *Psychopharmacology* **112**, 461–466 (1994).
8. Turton, M. D. *et al.* *Nature* **379**, 69–72 (1996).
9. Zhang, Y. *et al.* *Nature* **372**, 425–432 (1994).
10. Tartaglia, L. A. *et al.* *Cell* **83**, 1263–1271 (1995).

1. May, R. M. & Nee, S. *Nature* **378**, 447–448 (1995).
2. May, R. M. *Phil. Trans. R. Soc. Lond.* **B330**, 293–304 (1990).
3. Savage, J. *BioScience* **45**, 673–679 (1995).
4. Solow, A. R., Mound, L. A. & Gaston, K. J. *Syst. Biol.* **44**, 93–96 (1995).
5. Brown, D. S. *Freshwater Snails of Africa and Their Medical Importance* 2nd edn (Taylor & Francis, London, 1994).
6. Altaba, C. R. thesis, Univ. Pennsylvania (1991).
7. Altaba, C. R. *Butl. Inst. Cat. Hist. Nat.* **60**, 23–44 (1993).
8. Boeters, H. D. *Arch. Moll.* **118**, 181–261 (1988).
9. May, R. *Nature* **347**, 129–130 (1990).

may be at least 150 species in total. Thus, the synonymy rate here is most probably below 5%.

These examples show that different groups may have widely different synonymy rates. Long lists of synonyms may be expected for large, easily collected organisms with extensive geographical ranges and conspicuous individual variation. Conversely, hard to find, minute species with restricted ranges and uninteresting external appearance are likely to have none or few. Synonymy rates are affected by changing species concepts and improved analytical tools, rather than by continuous, predictable error of taxonomists.

Estimates of global biodiversity should therefore not rely on the number of described species, as this parameter is too

uncertain to provide any consistent estimate. Clearly, there is a need for more updated, robust taxonomic revisions, but taxonomy is a scientific discipline relevant on its own, able to contribute significantly to the preservation of biodiversity^{3,9}. Because the true extent of diversity in these three groups of molluscs has been unrecognized until now, these animals have been largely ignored by conservationists. It is sad indeed that many of these species, often still undescribed, are threatened or already extinct.

Cristian R. Altaba

*Institut d'Estudis Avançats de les Illes Balears (CSIC-UIB),
Ctra. de Valldemossa, Km 7.5,
07071 Palma de Mallorca,
Illes Balears, Spain*

Solvent-forming genes in clostridia

SIR — The Weizmann process¹, in which the bacterium *Clostridium acetobutylicum* converts corn starch into acetone and butanol, was used during the First World War to produce the acetone needed for smokeless gunpowder. The loss of the Weizmann strain's capacity to produce solvents after repeated subculture (degeneration)² is still not understood from the molecular viewpoint³. We show here that the solventogenic genes^{4,5} encoding the enzymes required for acetone and butanol formation are carried on a 210-kilobase (kb) plasmid (pWEIZ), and that three degenerate strains lack both these solventogenic genes and pWEIZ.

To investigate the molecular basis of degeneration, we isolated three spontaneous degenerate mutants (WDS1, 2 and 3) of *C. acetobutylicum* ATCC 4259 (the Weizmann strain used in warfare⁶) through serial subculturing. None of the mutants produced any detectable amounts of butanol or acetone, nor did they sporulate. DNA from the wild-type and mutant strains was prepared in agarose plugs and analysed by pulsed-field gel electrophoresis (PFGE).

Below a band of sheared chromosomal DNA in our gels, we always observed an extra band at 210 kb in the wild-type strain which was absent from all the degenerate mutants (see figure). DNA from the Weizmann strain digested by *Sma*I, *Apa*I or *Sst*II (see figure for the *Sma*I digest only) has a considerably higher-intensity 210-kb band than has the uncut DNA, whereas there is no 210-kb band for WDS1 DNA digested by the same enzymes. Because circular DNA cannot enter the gel in PFGE⁷, our data demonstrate: first, that *C. acetobutylicum* Weizmann contains a 210-kb plasmid (linearized by *Apa*I, *Sma*I and *Sst*II); second, that a small portion of it is linear in uncut DNA preparation; and third, that it is lost in the degenerate strains.

The genes encoding the enzymes required for the last steps of acetone and butanol production are clustered in the *sol* operon locus^{4,5}. To check whether these genes are carried on the plasmid, we used as a probe *ctfA* and *ctfB* genes (from *C. acetobutylicum* Weizmann), amplified by the polymerase chain reaction (PCR), encoding the two subunits of the

acetoacetyl-CoA: acetate/butyrate:CoA-transferase, one of the two enzymes involved in the final steps of acetone formation. We obtained a hybridization signal with the 210-kb linearized plasmid forms of *C. acetobutylicum* Weizmann, but no signal with WDS1 (see figure), indicating that the plasmid carries the *sol* operon locus. This plasmid was named pWEIZ.

Our finding raises an obvious question: what is the natural selective pressure for the maintenance of pWEIZ? The answer is probably linked to the fact that all the *C. acetobutylicum* strains that have lost pWEIZ also do not sporulate. It is tempting to speculate that an essential gene(s) needed for sporulation might be carried by the plasmid.

Emmanuel Cornillot

Philippe Soucaille

INSA, Centre de Bioingénierie

Gilbert Durand,

Complexe Scientifique de Rangueil,

F-31077 Toulouse Cédex, France

1. Weizmann, C. UK Patent 4845 (1915).
2. Bahl, H., Andersh, W., Braun, K. & Gottschalk, G. *Eur. J. appl. Microbiol. Biotechnol.* **15**, 201–205 (1982).
3. Woods, D. R. *Trends Biotechnol.* **13**, 259–264 (1995).
4. Petersen, D. J., Cary, J. W., Vanderleyden, J. & Bennett, G. N. *Gene* **123**, 93–97 (1993).
5. Fischer, R. J., Helms, J. & Dürre, P. *J. Bact.* **175**, 6959–6969 (1993).
6. Keis, S., Bennett, C. F., Ward, V. K. & Jones, D. T. *Int. J. syst. Bact.* **45**, 693–705 (1995).
7. Beverley, S. M. *Nucleic Acids Res.* **16**, 925–939 (1995).

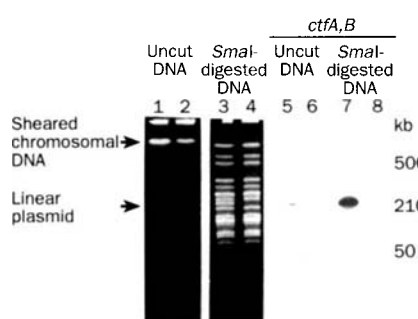
Mammal bones in Dominican amber

SIR — When and how did land vertebrates colonize the Greater Antilles, West Indies, during the Cenozoic? Were colonization events sporadic and fortuitous, or were they tightly clustered in time, suggesting the operation of a common cause (see ref. 1)? Fossils should help with these enquiries, but discoveries in conventional palaeontological contexts on these islands are exceedingly rare².

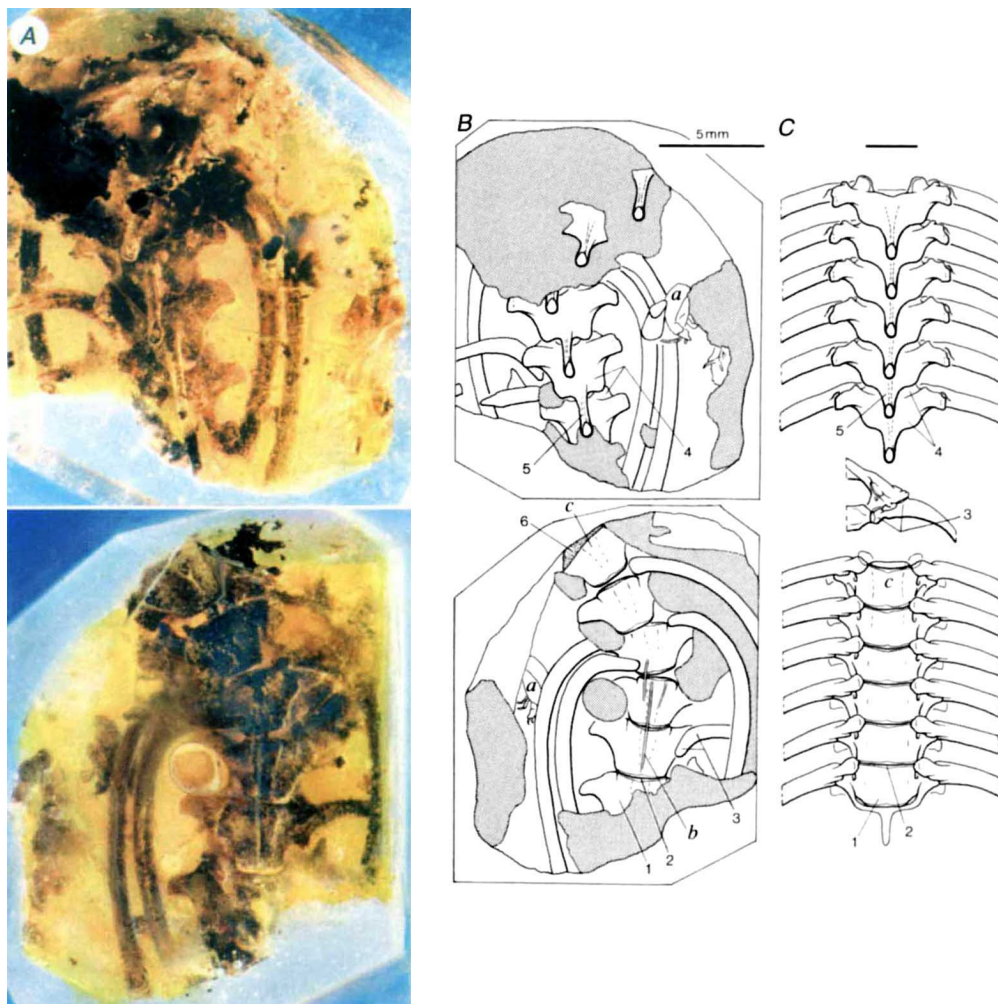
To a certain extent, this deficiency is made up by finds in Dominican amber, specimens of which occasionally contain small lizards and frogs. However, evidence for the presence of larger vertebrates, especially birds and mammals, has so far been limited to isolated (and rather undiagnostic) feathers and hair strands. We now report the discovery of the first mammal bones in Dominican amber (*A*, *B* in the figure), the identification of which provides additional evidence for the view³ that most mammalian colonizations were clustered early in the Tertiary.

The amber piece, containing six vertebrae and portions of several ribs in partial articulation, is reliably reported to have been collected from the La Toca group of amber mines in the La Cumbre region of the northern Dominican Republic. It lacks features indicative of forgery⁴, and we conclude that it is authentic. Recent re-analy-

Ethidium bromide-stained unrestricted (lanes 1, 2) and *Sma*I-digested (lanes 3, 4) DNA from *C. acetobutylicum* Weizmann (odd lane numbers) and WDS1 (even lane numbers) after separation by PFGE. The 1% agarose gel was run in $\times 0.5$ Tris-borate EDTA buffer for 18 h at 200 V (6 V cm^{-1}) using a linear ramp of pulse time from 10 to 50 s. Autoradiogram obtained, after hybridization with the PCR-amplified *ctfA* and *B* genes used as a probe, for unrestricted (lanes 5, 6) and *Sma*I-digested (lanes 7, 8) DNA from *C. acetobutylicum* Weizmann and WDS1. DNA was blotted on Hybond N⁺ (Amersham). The probe was labelled by random-priming with [α -³²P]-dATP and purified on a Microspin S200 (Pharmacia) column. Hybridization was performed at 65 °C in $5 \times \text{SSC}$, $5 \times \text{Denhardt}$, 0.5% SDS. Blots were washed at room temperature twice for 10 min in $2 \times \text{SSC}$ 0.1% SDS and twice for 30 min in $0.1 \times \text{SSC}$ 0.1% SDS.



A, B, Axial skeleton (AMNH DR 14627), probably of a solenodontid insectivore, embedded in amber from La Toca, northern Dominican Republic, in dorsal (top row) and ventral (bottom) aspects. C, Thoracic vertebrae 3–8 of extant Hispaniolan insectivore *Solenodon paradoxus* (AMNH 212911), in same aspects. Numbers identify specific features which, in combination, establish that the La Toca specimen is mammalian: 1, centra semi-cylindrical, wider than long, with a slight midsagittal keel (c); 2, intervertebral articulations acodelous (biplanar); 3, ribs with two articular facets (bicapital condition) and no conspicuous bony processes for muscle attachment; 4, articular apophyses on neural arch limited to pre- and post-zygapophyses; 5, neurapophyses comparatively long; and 6, internal bony architecture of fine-grained cancellous tissue, lacking pneumatic spaces or notochord apertures (as seen in fractured vertebra). Inset in C illustrates typical bicapital rib (feature 3) of *Solenodon*, seen in La Toca specimen and in mammals generally. Specific features of *Solenodon* and *Nesophontes* seen in the La Toca specimen (broad centrum, mid-sagittal keels, flaring diapophyses, wide intervertebral notch, strongly projecting neurapophyses) do not occur in this combination in bats, sloths, primates or rodents. Other inclusions in the amber specimen include a mordellid beetle (a), a cecidomyid gall midge, and small masses of fungi and amorphous material (shading in B) which may cover remnants of tissue from the original animal. Thin strips (b), apparently composed of dense connective tissue, may be remnants of the original ventral longitudinal ligament of the spinal column.



sis⁵ of criteria for amber dating indicates that amber from this region can be no older than Late Oligocene/Early Miocene. Sampling for DNA — even using minimally destructive techniques — was not regarded as feasible in view of the specimen's fragility and uniqueness.

Structural details of the vertebrae and ribs (features 1–6 in the figure) indicate that the specimen is definitely mammalian. Among mammalian groups with known Antillean representation, the bats, primates, rodents and sloths can be excluded on grounds of morphology, size or both factors. On the other hand, resemblances to Solenodontidae (endemic Antillean

lipotyphlans) are strong enough to warrant the conclusion that the La Toca specimen represents a small, *Nesophontes*-sized insectivore (about 150 g). The specimen shows less resemblance to certain marsupials, but metatherians are not known to have reached the Greater Antilles.

The earliest plausible date for mammalian colonization of these islands may be set at about 42 million years ago (Late Eocene), when the northern Greater Antilles first became permanently sub-aerial as one or more large landmasses^{6,7}. Recent palaeontological discoveries^{2,3} establish that megalonychid sloths arrived on the islands before 33–34 million years

ago (Early Oligocene); minimum ages for other major clades of Antillean land mammals represented by fossils fall within Late Oligocene/Early Miocene. This evidence is consistent with the proposition that there was a peak colonization period for mammals during the Late Palaeogene or earliest Neogene, defined by a 'window' perhaps only 10–20 million years wide (and conceivably much narrower). It is possible that these colonizations had a common cause, such as an evanescent landbridge formed by the Aves Rise^{2,8}, but conclusive evidence is lacking. In contrast to the mammalian palaeontological evidence, immunological distance data⁹ appear to show that amphibian and reptile colonizations occurred sporadically over much of the Cenozoic, with no temporal optimum (but see ref. 10).

R. D. E. MacPhee
Department of Mammalogy,
David A. Grimaldi
Department of Entomology,
American Museum of Natural History,
New York, New York 10024, USA

- Williams, E. E. in *Biogeography of the West Indies: Past, Present, and Future* (ed. Woods, C. A.) 1–46 (Sandhill Crane, Gainesville, 1989).
- MacPhee, R. D. E. & Iturralde-Vinent, M. A. *Am. Mus. Novit.* **3094**, 1–13 (1994).
- MacPhee, R. D. E. & Iturralde-Vinent, M. A. *Am. Mus. Novit.* **3141**, 1–30 (1995).
- Grimaldi, D., Shadrinsky, A., Ross, A. & Baer, N. S. *Curator* **37**(4), 251–274 (1994).
- Grimaldi, D. in *Amber, Resinites, and Fossil Resins* (eds Anderson, K. B. & Crelling, J. C.) 203–217 (Am. Chem. Soc. Symp. Ser. 617, Washington DC, 1995).

- Iturralde-Vinent, M. A. *J. Petrol. Geol.* **17**, 39–70 (1994).
- Lewis, J. F. et al. in *The Geology of North America Vol. H, The Caribbean Region* (eds Dengo, G. & Case, J. E.) 77–140 (Geol. Soc. Am., Denver, 1990).
- Holcombe, T. L. & Edgar, N. T. in *Biogeographical Aspects of Insularity* (ed. Azzaroli, A.) 611–626 (Accademia Nazionale dei Lincei, Rome, 1990).
- Hedges, S. B., Hass, C. A. & Maxson, L. R. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1909–1913 (1992).
- Page, R. D. M. & Lydeard, C. *Cladistics* **10**, 21–41 (1994).

Travels in ancient lands

Jeremy A. Sabloff

In Search of the Old Ones: Exploring the Anasazi World of the Southwest. By David Roberts. *Simon and Schuster*: 1996. Pp. 271. \$24.

PUBLIC interest in archaeology seems to be at an all-time high. Across the globe, archaeological materials are featured in newspapers, magazines, books and television programmes and on videos and even compact discs. Archaeology is further blessed (although some academics might say cursed) by growing numbers of amateur archaeologists who are becoming more actively involved with the field. Such pursuits run the gamut from participation in carefully run excavation programmes to the occasional visit to museums to — unfortunately — the looting of ancient sites.

One of the most time-honoured (and honourable) forms of amateur activity is the exploration of previously unknown or little known archaeological sites. The melding of wilderness adventure and archaeological discovery frequently results in publications that range from travelogues to detailed accounts of archaeological finds. David Roberts's modern travels across the rugged landscape of southwestern North America are an excellent example of the best of this amateur tradition.

Roberts is a professional writer and an avid hiker. Here he recounts his visits to a wide variety of renowned and relatively unknown architectural remains of the pre-Columbian ancestors of modern-day Pueblo peoples, who are known in the archaeological literature as the Anasazi. Roberts writes well, and his enthusiasm for exploring the outdoors, his interest in the environment and his appreciation of both the ancient and modern cultures of the American southwest make the book an entertaining and informative work that quickly absorbs the reader in the author's narrative. While

his introspective examination of his own reactions to the sites and people he encounters in his travels is quite engaging, his understanding of the intellectual and

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Window on the past — the remains of Anasazi villages or pueblos in Mesa Verde National Park. The ruins date from the Pueblo III period (twelfth to fourteenth centuries AD), which saw the fullest development and eventual decline of the cliff-dwelling culture.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

emotional issues that dominate southwestern archaeology today is particularly impressive.

Through his examination of the archaeological literature, interviews with knowledgeable individuals and travels in the region, Roberts is able to provide a

perceptive discussion of modern archaeological debates about the abandonment of many Anasazi sites at the close of the thirteenth century AD, as well as Native American views of this topic. He seems to favour the argument that environmental stress led to endemic warfare and the growing need for defence. Eventually, the ever-present conflict and the pull of the socially cohesive Kachina Phenomenon to the south led to the abandonment of much of the northern Anasazi area. But Roberts is careful to point out the variety of professional viewpoints on this topic and to indicate that there is still much disagreement.

The author also makes a strong case for the contributions of the Wetherill brothers, especially Richard Wetherill, at the end of the last century and the early part of this one, in helping launch the field of Anasazi archaeology, and dismisses claims that the Wetherills were nothing more than glorified pothunters. Roberts further offers some useful comments about the policies of the US National Park Service regarding the preservation of archaeological sites and public access to them, and the contradictions and problems faced by past government stewards and by interested tourists.

Although there are some inaccuracies and gaps in Roberts's coverage, occasional reliance on out-of-date archaeological information (on the Toltecs or the dating of the domestication of maize in Mexico, for example), or over-romanticism (as in the chapter on the "open museum"), the author has done his homework and is usually judicious in his discussions of contentious issues.

Both the general reader interested in exploration in general or the American southwest in particular and the academic will find this evocative book worthy of their attention. □

Jeremy A. Sabloff is at the University of Pennsylvania Museum of Archaeology and Anthropology, Philadelphia, Pennsylvania 19104-6324, USA.

B. Norman/Ancient Art and Architecture Collection

Susan Silberberg/Ancient Art and Architecture Collection



BLUE sky research — this cloudless digital image of Earth was built by ARC Science Simulations using US Geological Survey data collected by the NOAA-11 weather satellite. It is the highest-resolution picture so far of our planet and to do justice to the detail would require a print 20-foot-wide at *Nature's* current resolution.

A prospect of time

Euan Nisbet

The Evolving Continents, Third Edition. By Brian F. Windley. Wiley: 1995. Pp. 526. £19.99, \$32.50 (pbk).

DOES one trim the old and interplant the new, or brutally cut the whole lot down and start again? There was until quite recently in Windsor Great Park a line of once fine but now decaying oak trees where rode America's last female head of state (her administration was, incidentally, very successful, as were those of the two previous women to head the government). That was until the present ranger of the land, the Duke of Edinburgh, decided to cut down the old, hollow, blasted oaks of Queen Anne's Ride (with their associated beetle fauna) in order to replant the entire avenue for the centuries to come. The local bureaucrats, having covered large areas of the surrounding land with asphalt, roared in indignation at the tree-cutting and demanded control, amid Wodehousian scenes of dawn felling. With some sorrow for the beetles evicted from their hollow homes (there are, fortunately, other aged trunks nearby), I have much sympathy for the duke. Instead of bequeathing an eloquent but dying straggle of foliage, he will leave to the future a vigorous and rewarding prospect (which, I hope, the bureaucrats will not asphalt over for a new highway to Virginia Water: Windsor Park remains green, although the sur-

roundings are as road-ridden as the stretch from Annapolis through Maryland to Virginia).

Brian Windley has had the same problem. Since before this generation of first-year students was born, his book has been a standard text in Earth-history classes, especially those that pay due respect to the Precambrian bulk of the planet's life. The first edition, in 1977, offered a pleasing prospect, a long view from the birth of the Earth to the present. Then, in 1984, for the second edition, he cut out some of the more decayed parts, pruned and interplanted assiduously. This time, for the third edition, he has boldly chain-sawn the lot, turned the whole view around so that it now goes from modern to old, and produced what is essentially a new book.

As with all new books, it has its problems. Some of the diagrams, straight from research papers, look cheap, and some reproduce badly. In such a large new book, which must have taken a while to grow, some of the weeds are already apparent. But the 2,200 references are the oaks that make the ride through the book so valuable. Instead of an eloquent but dying straggle of verbiage, Windley presents a vigorous and rewarding prospect to the past. In all, for a senior class wishing to learn from the history of our planet, this is a useful book, laid out not with a planner's well-paved and sign-posted barrenness of the moment, but with a duke's long eye to time. □

Euan Nisbet is in the Department of Geology, Royal Holloway College, University of London, Egham, Surrey TW20 OEX, UK.

Staying out of trouble

Peter W. Lipman

Natural Hazards. By David Chapman. Oxford University Press: 1995. Pp. 174. £12.50, \$15 (pbk).

WHAT are the full spectra of natural hazards, how do they affect humans and in what ways can risk mitigation and management be effective? Paradoxically, as we become more knowledgeable about natural catastrophes and develop better strategies to lessen the damage they wreak on society, they are becoming increasingly harmful in terms of loss of life and damage to property. This is largely a result of world population growth, increased vulnerability of economically and technologically complex societies, limited resources for low-income populations and mismanagement of the environment.

In this succinct volume, David Chapman, a geographer at the University of Sydney, discusses such broad topics of global importance from a wide variety of perspectives, characterized by a deliberately Australian flavour. Treatment of biological hazards ranges from the threat of large carnivores to allergic reactions to plants, and meteorological phenomena related to storms, floods, droughts and wild fires are tackled hand in hand with geological subjects such as earthquakes, volcanoes and landslides. The focus is on frequent and rapid catastrophes. Slower hazards, such as global effects of long-term climate change or more locally important effects such as damage to buildings from swelling clays, are excluded, even though they too can have far-reaching long-term effects. In the United States, swelling clays make up the most economically damaging geological hazard, outranking more spectacular catastrophic events such as earthquakes and volcanic eruptions.

In his discussion of management models for mitigation of natural hazards, Chapman emphasizes rational approaches to decision-making based on cost-benefit analysis. But much recent experience casts doubt on whether we can assume that affected populations and civil authorities will use scientific and economic data rationally. Psychological, economic and sociological issues important in risk analysis and hazard mitigation are barely mentioned in the book. Few people are entirely rational about risk analysis; most tend to accept familiar risks more readily than similar but infrequent risks. They are more willing to accept the higher risk of car travel than plane travel, or the statistically greater

health risk from inhaling tobacco smoke than from eating British beef. Infrequent but spectacularly damaging earthquakes and volcanoes make headlines; swelling clays do not.

Mitigating risk from natural hazards involves effective communication and mutual understanding of methods and priorities by technical advisers (scientists, economists, land-use planners) and civil authorities legally responsible for making decisions. These authorities must also take into account the urgent demands of existing land use, local economics and election politics. An economic factor of considerable importance today in the United States is the influence of the

availability and cost of insurance on decisions about land use and investment; reduced availability of property and liability protection in some high-risk areas has had profound effects on land value, economic activity and patterns of urbanization. Improved communication among scientists, economic managers, civil authorities and the public may well offer the largest cost-benefit ratio for society in the near-term mitigation of natural hazards. □

Peter W. Lipman is in the Volcano Hazards Program, US Geological Survey, 345 Middlefield Road, Menlo Park, California 94025, USA.

Selfishness in a cooperative world

Laurent Keller

Evolution of Social Insect Colonies: Sex Allocation and Kin Selection. By Ross H. Crozier and Pekka Pamilo. Oxford University Press: 1996. Pp. 306. £39.50, \$62 (hbk); £19.95, \$32 (pbk).

THERE is something distinctly odd about social insects. They provide some of the most fascinating examples of altruistic behaviour, with a worker caste whose individuals forgo their own reproduction to aid in the reproduction of the queen, as well as many other examples of self-sacrifice, including the evolution of 'kamikaze' weapons such as detachable stings and exploding abdomens used in defence of the colony. Yet life within the colony is not always as harmonious as it may at first appear. Individuals may use Machiavellian strategies to favour their own interest, and lethal fights are not uncommon. It is this complex dynamic between conflict and cooperation that Ross Crozier and Pekka Pamilo consider in this excellent book.

The authors convincingly demonstrate that kin selection is the main selective force responsible for the evolution and maintenance of sterile castes and altruistic behaviour in ants, bees, wasps and termites (the major groups of social insects). The general principle of kin-selection theory is that individuals can pass on copies of their genes not only by producing offspring but also by helping kin such as siblings to reproduce. To maximize their fitness, colony members should therefore favour individuals that are more closely related, and this provides the raw material for many potential conflicts within the colony. For example, the haplodiploid sex-determination system means that workers are more closely related to their sisters than to their brothers in ants and other Hymenoptera. So workers should favour a female-biased sex-ratio

investment by their colony. By contrast, because queens are equally related to their sons and daughters, they should favour an equal investment in both sexes. Queen-worker conflict over sex ratio is therefore predicted, and evidence indeed indicates that workers cannibalize males — their brothers — to manipulate colony sex ratio in their favour.

Two other important types of conflict between colony members concern the

tion in the number of queens per colony and the mating frequency of queens — the main factors affecting colony genetic structure — should affect the nature of within-colony conflicts.

My only reservation about this book stems from its long gestation, which has resulted in several recent theoretical concepts and empirical findings being omitted or mentioned only briefly. For instance, several empirical studies of colony-level variation in sex ratios are only briefly treated and there is no mention at all of skew models, although these models provide powerful tools to investigate reproductive conflicts among colony members, by considering simultaneously the effects of relatedness, ecological factors and intrinsic benefits of cooperation.

I was expecting much overlap between this book and Andrew F. G. Bourke and Nigel R. Franks's recent *Social Evolution in Ants* (Princeton University Press, 1995). To my surprise, that is not the case. For one, Bourke and Franks's book deals only with ants, whereas Crozier and Pamilo's covers all social insects. The approach of the two books is also quite different. Crozier and Pamilo develop a new theoretical framework for analysing kin conflicts within insect societies. A lot of this work has been published in recent and already influential papers. Bourke and Franks use a less mathematical approach

and provide a more general overview of empirical studies on social behaviour and sex ratio. The two books are therefore complementary. Bourke and Franks's book will be preferred for those looking for broad synthesis of the fascinating world of insect sociality, whereas Crozier and Pamilo's book is an excellent resource for those interested in a mathematical approach to kin conflicts and sex-ratio theory. The use of elegant kin-selection models also makes Crozier and Pamilo's book highly suitable for a course on theoretical modelling.

It took millions of years for social insects to attain their complex social organi-

zation. The rapid empirical and theoretical progress reported in this book provides some hope that it might take less time to attain a good understanding of how insects have evolved their sophisticated sociality. □

Laurent Keller is at the Institut de Zoologie et d'Ecologie Animale, University of Lausanne, 1015 Lausanne and the Zoologisches Institut, Bern University, Switzerland.



The solitary masarine wasp *Ceramius clypeatus*. From *The Pollen Wasps: Ecology and Natural History of the Masarinae* by Sarah K. Gess. Harvard University Press, £31.50, \$49.95.

identity of individuals reproducing in the colony and the way in which resources should be allocated between reproductive and non-reproductive functions: that is, between the production of reproductive versus worker individuals. Crozier and Pamilo use formal kin-selection models to investigate how these conflicts are affected by variation in the genetic structure of the colony. Their analyses yield interesting predictions about how varia-

Underdetermining the truth

Peter Lipton

Beyond Positivism and Relativism: Theory, Method and Evidence. By Larry Laudan. Westview: 1996. Pp. 277. \$59.95 (hbk); \$22.95 (pbk).

FOR scientists, data often provide a daunting constraint on theory: the challenge is to find even one theory that fits all the relevant evidence. By contrast, philosophers, particularly those of a sceptical inclination, have been impressed by the weakness of the empirical constraint. One of the most powerful weapons in the sceptic's arsenal is the argument from empirical equivalence. The sceptic grants the truth of whatever we choose to count as our evidence, but denies us knowledge of the claims we infer from it, on the grounds that there will always be contrary claims that are also compatible with that evidence. Thus, in the seventeenth century, René Descartes famously argued that the evidence of the senses could not possibly yield any knowledge of the external world, because that evidence is always compatible with the contrary hypotheses that we are just dreaming or under the influence of a malign and powerful deceiver.

Arguments from empirical equivalence have also attracted philosophers of science in the present century. For example, Willard van Orman Quine, the distin-

guished philosopher and logician, has claimed that all scientific theories are 'underdetermined' by the evidence. However much data a theory may have in its favour, there will always be in principle innumerable other theories, inconsistent with the first but consistent with these data. The underdetermination thesis has opened the door to philosophical accounts that deny realism, the view that science is capable of generating increasingly accurate representations of a largely unobservable world. According to some philosophers, the moral of underdetermination is that scientific theories can only be calculation devices for generating predictions; according to others, the slack that the data leave must be taken up by varying social and political factors, so the only defensible view of science is wholesale relativism.

Several of the essays in this stimulating collection by Larry Laudan, a prolific and influential philosopher of science, aim to undermine underdetermination. Laudan argues that the underdetermination thesis is ambiguous, and that the acceptable version is also epistemically innocuous. All that version shows is that theories can never be deduced from the data, which ought to be a truism. The support that evidence provides for theory will never amount to a proof. A threatening version of the underdetermination thesis would have to claim that, however extensive the evidence, there will always be innumerable competing theories that would be equally well supported by that evidence, a position that Laudan claims is implausible, and undefended by the friends of underdetermination. Laudan also argues that the notion of empirical equivalence is ill-defined, because the class of possible evidence that could bear on a theory varies over time. The auxiliary statements that may be used in concert with a theory to generate testable predictions will change; so will the class of observable states of the world, with innovations in instrumental technology. In this way, two old theories that could not be evidentially distinguished on the basis of naked-eye observation may well have become distinguishable with the invention of the microscope.

Given Laudan's robust criticism of underdetermination, one might expect him to offer a realist account of science. As other chapters in his book show, however, this is very far from the truth. He denies that scientific development is cumulative: new theories do not in general explain everything that their dethroned predecessors explained. For example, Descartes's vortex theory of planetary motion explained why all the planets move in the same direction, whereas Newton's theory did not. He also claims that both the aims and methods of science vary within and between

scientific communities. Finally, although various different and incompatible scientific aims are legitimate, the realist's aim of discovering the truth about the world is not.

Laudan scorns relativism, which he regards as the occupational disease of recent philosophy of science, with Thomas Kuhn cast as a particularly virulent influence. Given Laudan's own position, however, one could be forgiven for thinking that he has himself been infected. Laudan nevertheless maintains that his own account is immune to relativism. Legitimate aims and methods may vary, and truth is not on the cards; but it is supposed to be an objective matter whether a particular method is a reliable means for realizing a particular aim. Laudan claims that the effectiveness of a scientific method is itself susceptible to empirical investigation, by testing for the correlation between the application of the method and the realization of the aim, in particular episodes in the history of science. Moreover, although Laudan firmly rejects a cumulative view of the development of science, he holds that science is objectively progressive, because later theories solve more problems than their predecessors, even though some old solutions are dropped without replacement.

Laudan's pluralistic account of science is genuinely different from simple relativism, but his presentation is open to challenge. Although their positions are not the same, Laudan misrepresents Kuhn in a way that exaggerates the contrast. In his classic *Structure of Scientific Revolutions* (University of Chicago Press, 2nd edn, 1970), Kuhn writes: "Later scientific theories are better than earlier ones for solving puzzles in the often quite different environments to which they are applied. That is not a relativist's position, and it displays the sense in which I am a convinced believer in scientific progress."

Laudan is also at one with Kuhn in the rejection of the realist view of truth as a legitimate aim of science. Although the issues are complex and Laudan has elsewhere provided direct arguments against realism, his criteria for legitimate aims may beg the question against truth. He holds that a pairing of method and aim is legitimate only if it can be empirically certified by finding empirical correlations that show the method to be effective. But he construes this test of method in a way that rules out truth from the start, on the grounds that we cannot observe whether a theory is true, and so cannot directly check for a correlation between any method and that aim. Truth is in this way disqualified before the race has begun. □

Peter Lipton is in the Department of History and Philosophy of Science, University of Cambridge, Free School Lane, Cambridge CB2 3RH, UK.

New in paperback

Dance for Two by Alan Lightman. Pantheon, \$12. A collection of essays examining the way literary and scientific concerns can be brought into harmony.

The Ghost of the Executed Engineer: Technology and the Fall of the Soviet Union by Loren R. Graham. Harvard University Press, £7.95. The life of the engineer Peter Palchinsky in Stalinist Russia.

Children of Choice: Freedom and the New Reproductive Technologies by John A. Robertson. Princeton University Press, \$16.95, £13.95. Examines the repercussions of developments in reproductive techniques.

Physical Origins of Time Asymmetry edited by J. J. Halliwell, J. Pérez-Mercader and W. H. Zurek. Cambridge University Press, £24.95, \$39.95.

Universes by John Leslie. Routledge, £10.95. A philosophical look at the question 'Why does our Universe exist?'

Chemistry Imagined: Reflections on Science by Roald Hoffmann and Vivian Torrence. Smithsonian Institution Press, £15.50.

Essential flexibility in the T-cell recognition of antigen

Gilbert J. Kersh & Paul M. Allen

$\alpha\beta$ T cells specifically recognize a ligand composed of a peptide bound to a self-major-histocompatibility-complex molecule, but the recognition of slightly altered ligands by T cells can lead to a partial activation. This flexibility is crucial for T-cell development and can have both beneficial and harmful effects on peripheral T cells.

"He that wrestles with us strengthens our nerves, and sharpens our skill. Our antagonist is our helper"¹. With this statement, the eighteenth century British statesman and philosopher Edmund Burke was unknowingly remarking about a feature of the immune system, the recognition of suboptimal ligands by T cells. Although the main event in a specific immune response involves the recognition of antigen by T cells, a series of recent studies have shown that T cells do not simply recognize antigen and respond in a binary manner, either being on or off, but can productively recognize suboptimal ligands and respond in a variety of ways. These findings have implications for many aspects of T-cell biology and are the subject of this review.

T-cell recognition of antigen

Each individual T cell expresses a clonally distributed receptor (T-cell antigen receptor, TCR) which recognizes a ligand composed of an 8–15-amino-acid-long antigenic peptide bound to a self-major-histocompatibility-complex (MHC) molecule^{2,3}. Thus, a T cell does not directly recognize a soluble antigen, but rather recognizes an antigen displayed on the surface of an antigen-presenting cell (APC) or a target cell.

The TCR is crucial for the development, differentiation, and effector functions of T cells. The TCR itself does not have any intrinsic signalling capacity, but is part of a multichain complex which links it to multiple intracellular signalling pathways^{4–6}. A primary event in T-cell activation involves the phosphorylation of the CD3 and TCR ζ -chains by Src family kinases and the subsequent activation of the ZAP-70 kinase⁷. The signals generated by the activation of ZAP-70, along with other intracellular signalling pathways also stimulated by TCR recognition, are then integrated by the T cell, leading to full activation⁸. Although the precise reason why the immune system has developed such a highly intricate signalling mechanism is not known; one can make the teleological argument that because of its mere existence, this multicomponent signalling complex must be an essential feature of an effective anticipatory cell-mediated immune system.

The evolution of an effective T-cell-mediated immune system had to fulfil several important criteria. T cells must be able to recognize a panoply of foreign antigens which they have never seen, be highly specific, be able to respond to a small amount of antigen, and not recognize the normal host. The requisite specificity could easily have been obtained by a high-affinity receptor; however, several studies indicate that the TCR has a relatively low affinity for its ligand, with a micromolar dissociation constant (K_d)^{9–12}. Furthermore, on any given APC only a small proportion of the MHC molecules are expressing the same antigen, because APCs do not distinguish between self and foreign antigens, but are constantly sampling their environment, the majority of which is composed of self molecules. The self/non-self discrimination process is therefore the responsibility of the T cells, occurring mostly during the development of T cells in the thymus.

The crystal structures of peptide–MHC complexes have shown that only three to four side chains of the peptide are accessible for

recognition by the TCR^{13–16}. Thus, a TCR recognizes a bimolecular surface, much of which is composed of the self MHC molecule, with the peptide contributing key electron density in the centre. With the limited number of side chains being recognized, one could imagine that similar electron densities could be achieved by several different peptides. Functional evidence in support of this has now been reported with non-homologous peptides being able to stimulate a single TCR^{17–19}.

Models for T-cell recognition of antigen

Given the relatively low affinity of the TCR and the limited number of ligands, the basis for exquisite TCR specificity is not inherently obvious. Two recent complementary models together provide a theoretical and experimental basis for the T-cell recognition of a foreign antigen, and explain how T cells can productively interact with suboptimal ligands^{20,21}.

One model for TCR signal transduction has been proposed that is based upon the concept of kinetic proof-reading, known to be a key mechanism that preserves the fidelity of protein and DNA synthesis²¹. The basis of this model is that the TCR and its associated molecules require an accumulation of phosphorylation events after ligand binding to transmit a positive signal. This requirement for multiple phosphorylations introduces a temporal lag between the binding of the peptide–MHC ligand and TCR signalling. The mechanistic complexity of TCR signal transduction is therefore a way in which a T cell can survey many different ligands and specifically respond to ligands with a sufficiently high affinity while ignoring those with only a slightly lower affinity.

Another model based on experimental findings can explain how a T cell can be stimulated with a limited number of ligands²⁰. This serial triggering model proposes that a single peptide–MHC ligand engages multiple TCRs in a serial manner. First, one peptide–MHC complex interacts with one TCR. This TCR then forms part of the TCR contact cap, it detaches from the ligand, permitting the ligand then to bind to another TCR. This repeated process results in the assembly of a sufficient number of TCRs in the contact cap for productive signalling to occur, even when there are only a limited number of ligands.

Partial T-cell activation by altered ligands

An appreciation of the subtleties in the T-cell recognition of antigen has recently been made because of the realization that T cells can interact productively with suboptimal ligands. Flexibility in TCR recognition of its ligand was initially established in a report describing a CD4⁺ Th2 clone that responded to a peptide–MHC ligand by proliferating and producing interleukin-4 (IL-4), but responded to a single amino-acid variant of the original peptide by producing IL-4 in the complete absence of proliferation²². This result demonstrated that the TCR could sense subtle changes in its ligand, and respond differently on the basis of recognition of the variant peptide, a phenomenon we have defined as partial T-cell activation²³.

The discovery that the TCR is not simply an on/off switch was

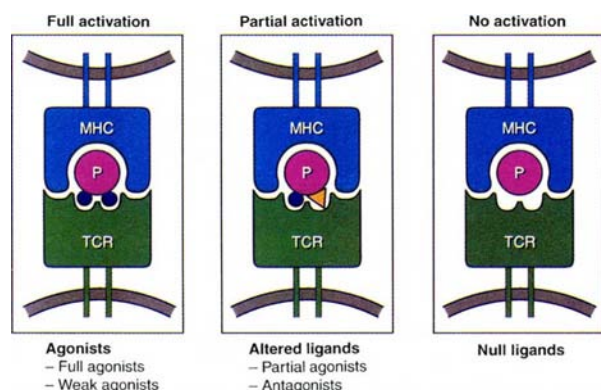


FIG. 1 Three possible outcomes of TCR interaction with a peptide-MHC ligand. Full activation is achieved when a TCR recognizes an agonist peptide (P) complexed with the proper MHC molecule. Ligands that induce all T-cell functions, but at higher concentrations than the agonist, are considered weak agonists. We refer to variants of the agonist ligands as altered ligands, and these can induce partial activation of the T cell. The majority of altered ligands are created by making single amino-acid changes in the peptide, and are therefore called altered peptide ligands. This category of ligands includes both partial agonist peptides and TCR antagonist peptides. The partial agonists are a subset of antagonists. The third class of ligands are those that induce no response from the T cell, and we refer to these as null ligands.

followed by a report showing that subtle variants of antigenic peptides could act as antagonists by specifically inhibiting the response of CD4⁺ helper T cells to the antigenic peptide²⁴. The effect was not based on the differential capacity of the peptides to bind the MHC molecule, nor was it due to simple competition for available MHC binding sites on the APC.

Subsequently, other studies have shown that recognition of sub-optimal ligands is a general characteristic of T cells^{25–31}. Peptides have been identified that act as antagonists of cytotoxicity mediated by CD8⁺ T cells²⁵. Other variant peptides have been found to inhibit the response of CD4⁺ T cells to bacterial superantigens³⁰. Furthermore, subtle changes in the ligand that influence the T cell need not be limited to changes in the peptide. A peptide presented on a mutant class II MHC molecule will induce a response that is different from the response induced when presented by the wild-type MHC molecule²⁶.

Another profound effect that altered ligands can have on T helper cells *in vitro* is the induction of a state of unresponsiveness or anergy^{32,33}. Previously it had been shown that T cells could be anergized by elimination of the costimulatory signals when the T cells were being antigenically stimulated^{34,35}. These anergic T cells could not be stimulated through their TCR, but still retained the ability to proliferate under the influence of IL-2. Similarly, it was shown that T cells can be exposed to an altered ligand and not proliferate or produce lymphokines, but are rendered anergic^{32,33}. The anergy induced by the altered ligand was not prevented by simply providing a costimulatory signal³², indicating that a different signalling pathway was being affected. Remarkably, the minor alteration of a single amino-acid residue had profound consequences for the T cells, being fully stimulated by the antigenic ligand, and anergized by the altered ligand.

The unifying feature of these studies is that subtle modifications within the peptide-MHC ligand result in partial T-cell activation. These modifications could be made in several different ways, including: directly altering a TCR contact residue of the peptide; changing an MHC contact residue of the peptide, which results in an allosteric change in a TCR contact residue; altering an amino-acid residue in the peptide which makes a change in the MHC molecule itself; or directly modifying residues of the MHC molecule which contact the TCR. Because of the exquisite sensitivity of the TCR, these changes in the composite ligand only need to be very minor, such as the alteration or repositioning of a single amino-acid side chain.

Taken together, these findings clearly show that a T cell can respond in a variable manner depending upon the ligand being recognized. Figure 1 shows a simplified model for how a TCR can interpret different ligands^{23,36–38}. Optimal-fit ligands that activate all functions of the T cell are referred to as agonists. Those ligands that are slightly less optimal, and require higher concentrations than the antigenic peptide to induce all T-cell functions, are called weak agonists. The second class of ligands are those that have a significant but suboptimal fit and induce partial T-cell activation. The general term 'altered ligands' describes these molecules,

which include partial agonists and antagonists. The majority of altered ligands fall into the category of altered peptide ligands (APLs), a term we use to describe those altered ligands that are produced by an amino-acid change in the antigenic peptide sequence. The third class of potential ligands contains those that have no fit with the TCR and result in no response at all from the T cell and are called null ligands.

Mechanism of partial agonist signalling

The observed biological responses indicated that partial agonists were inducing some T-cell signalling events. Direct examination of the proximal signals induced by partial agonists revealed that they were inducing a unique signal, indicated by a unique pattern of phosphorylation of the TCR ζ -chain and a failure to activate the ZAP-70 kinase^{39,40}. The strength of the signal delivered by partial agonists was less than the agonist; however, this was not simply a reflection of an overall weaker signal, but was a qualitatively different signal. Thus, the pattern of TCR ζ -chain phosphorylation was unique and not observed with any concentration of an agonist.

The engagement of the TCR by an agonist ligand induces multiple signalling pathways in a T cell, which when integrated in a coordinated manner lead to full activation⁴¹. These include the ζ /ZAP-70/NFAT pathway, the Ras pathway, and the costimulation pathway. Our current model is that in partial T-cell activation, some of these pathways are activated either partially or fully, whereas others are not activated at all⁴². Stimulation by an altered ligand results in a major decrease in the ζ /ZAP-70/NFAT pathway, whereas the Ras and costimulation pathways appear to be activated. The integration of these signals then leads to partial T-cell activation. According to this model, any stimulation through a limited subset of signalling pathways would lead to a similar phenotype. This is precisely what happens during the induction of anergy by stimulation in the absence of the costimulation pathway³⁵. Therefore, stimulation by a partial agonist results in the imbalance of the normal coordinate signalling pathways in a T cell, leading to partial T-cell activation.

How can the TCR perceive different ligands?

How can a ligand that differs very slightly from an agonist deliver a unique signal to the T cell? Many models have been proposed to answer this question^{21,36–38,42–44}. For simplicity we have grouped the possible explanations into the two models depicted in Fig. 2. The first model relates to the kinetics of the interaction between the TCR and its ligand and suggests that altered ligands deliver a unique signal to the T cell by providing less of the same type of interaction than an agonist ligand provides^{21,45}. In this type of model, the TCR has a reduced affinity for the altered ligand compared to the agonist because of an increased off-rate. This would result in a shorter interaction time between the TCR and its ligand, which would change the size and/or composition of the key signalling molecules in the TCR contact cap, resulting in a qualitatively different signal. In support of this model, off-rates

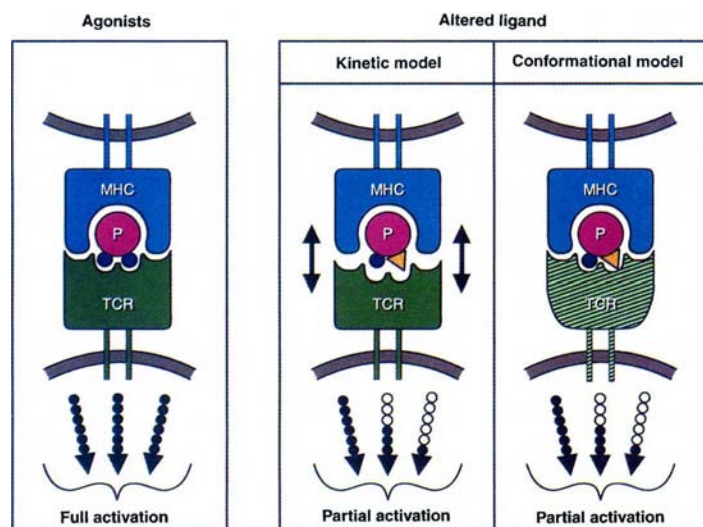


FIG. 2 How can the T cell tell the difference between an agonist and an altered ligand? An agonist ligand will induce a complete set of signals through the TCR, resulting in full activation of the T cell. Models for how an altered ligand can induce a partially activating set of signals fall into two categories. The kinetic type of model is that a different signal results from the altered ligand having less of the same type of interaction as the agonist. Recognition of the altered ligand is qualitatively the same as the agonist ligand, but is quantitatively less. In this model, a key difference between an agonist ligand and an altered ligand would be that the TCR would have a faster off-rate for the altered ligand than the agonist. The TCR interacts with the MHC for a shorter time and this leads to a different signal. In the conformational type of model, the APL recognition is qualitatively different from recognition of the agonist ligand. In this type of model a different signal results from a different structural basis for the TCR–ligand interaction. TCR signal transduction would involve some type of conformational change, and this change would be qualitatively different when the TCR recognizes an agonist as opposed to an altered ligand. This qualitative difference in ligand recognition would lead to a different signal.

have been found to be faster for some TCRs interacting with altered ligands compared to interaction with their corresponding agonist (ref. 45 and D. S. Lyons *et al.*, manuscript in preparation). Alternatively, the second model contends that^{43,46} the conformation of the TCR is different, depending on whether it recognizes an agonist or an altered ligand. The different conformational states of the TCR would then be able to induce unique signals. Several studies support the conformational model, but so far there is no direct evidence showing a conformational change in the TCR⁴⁶. Whether either or both of these models are correct must await structural and biophysical studies that will elucidate exactly how the TCR recognizes its ligand.

Relevance of recognition events for T cells

In vitro studies using cloned T-cell lines have definitively shown that T cells can interact productively with suboptimal ligands. We contend that this is an essential part of the life and function of T cells, especially in their development in the thymus.

Thymic development. The thymus produces a peripheral T-cell repertoire that can respond to foreign but not self antigens⁴⁷. Thymic development of T cells occurs in the absence of foreign antigen, but selects a population of T cells that can recognize potential foreign antigens bound to self MHC molecules. Because the MHC genes are highly polymorphic, each individual will have their own set of MHC molecules, and needs to select a TCR repertoire that is compatible with their particular MHC haplotype. This process is termed positive selection, and we contend that it must be the primary driving force behind the evolution of productive T-cell interaction with suboptimal ligands.

T cells developing in the thymus take different developmental paths depending upon the fit of self peptide–MHC complexes recognized by the TCR (Fig. 3). If these cells do not recognize self peptide–MHC at all, they will die from neglect without further differentiation. To be rescued from this cell death, thymocytes must be positively selected by recognition of an appropriate fit self-peptide–MHC complex. For some thymocytes, recognition of an optimal fit self-peptide–MHC induces cell death, a process called negative selection, which results in tolerance to self antigens. It is still not clear how some ligands in the thymus induce cell death and others induce positive selection, nor why T cells that must recognize self-peptide–MHC to escape cell death in the thymus do not respond to that same self-peptide–MHC in the periphery. These enigmas may possibly be resolved by the discovery that T cells can in some way be stimulated by suboptimal ligands.

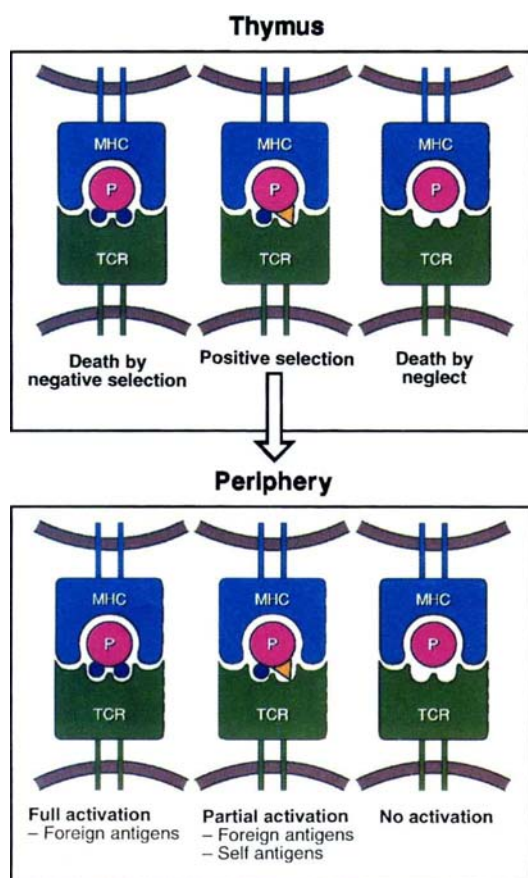


FIG. 3 T cells can experience different ligands both in the thymus and in the periphery. In the thymus, recognition of an agonist ligand leads to full activation and negative selection by activation-induced cell death, whereas no recognition of ligand leads to programmed cell death. Recent data suggests that positive selection and export of functional T cells to the periphery occurs after the TCR on thymocytes recognizes a low-avidity ligand, and some form of partial activation ensues. APLs seem to be the most efficient at providing this low-avidity interaction. In the periphery, agonist ligands for the TCR are derived from foreign antigens, and lead to full activation of T-cell function. T cells also encounter altered ligands that can be derived from either endogenous proteins or foreign antigens. Recognition of altered ligands by peripheral T cells may lead to anergy, may provide a virus with the ability to antagonize a CTL response, and may change patterns of cytokine production. Null ligands have no effect on peripheral T cells.

Important insights into the role of peptides in positive selection were gained from *in vitro* studies using TCR transgenic systems in which the positive selection of a single TCR could be examined^{49–51}. These seminal studies show definitively that positive selection of T cells can be mediated by specific peptides that are related to the agonist ligands. Altered peptide ligands of the agonist efficiently induced positive selection, whereas unrelated peptides were very inefficient. Thus, by extrapolation, positive selection occurs normally by the recognition of sub-optimal ligands from the endogenous peptide repertoire. Clearly, the interactions that induce positive selection will have a lower avidity than the interactions of peripheral T cells with foreign agonists. Multiple variables affect this overall avidity, including the affinity of the TCR for the ligand, the density of the ligand, and the contribution of coreceptor and other adhesion molecules^{52,53}. The identification of which self peptides are inducing positive selection, how they relate to the actual agonist, and how many T cells can be positively selected by a single peptide are important unresolved issues. Based on the highly flexible TCR recognition already demonstrated, we would contend that an individual ligand could select large numbers of TCRs (hundreds of thousands), and an entire repertoire may only require a few peptides for selection. Therefore, we conclude that the ability of a T cell to respond productively to suboptimal ligands is an essential part of the development of T cells.

One complication with T cells being selected on self-peptide–MHC complexes in the thymus is that the same ligands are also expressed in the periphery. How can T cells be selected on a self-peptide–MHC ligand and yet tolerate it in the periphery? The explanation appears to be that thymocytes have a lower threshold of activation than peripheral T cells^{54–56}. A suboptimal ligand in the thymus can therefore induce positive selection of a T cell, whereas in the periphery it is ignored.

Peripheral T cells. We propose that T-cell recognition of sub-optimal ligands is necessary for the generation of an anticipatory immune system. Peripheral T cells under normal circumstances do not productively interact with suboptimal ligands, but the *in vitro* studies clearly show that they have retained this capacity. We contend that under limited but attainable circumstances, peripheral T cells can undergo partial T-cell activation, having both beneficial and harmful outcomes.

Achilles' heel of T cells. The ability to interact productively with suboptimal ligands could have deleterious effects to the host and

therefore could be an 'Achilles' heel' of the immune system. There have now been three reports that viruses may exploit this Achilles' heel of T cells as one of their mechanisms to avoid immune elimination. Naturally occurring viral variants of HIV and hepatitis B virus CTL epitopes *in vivo* can act as antagonists of the cytolytic response of T cells to the antigenic epitope *in vitro*^{57,58}. The fact that viruses expressing antagonist epitopes can be found in infected individuals suggests that these viruses have a selective growth advantage over mutant viruses whose epitopes do not interact with the viral-specific TCR at all. A general model is that an antagonistic variant has the ability to shut off a T cell that would otherwise lyse the infected target. This would spare the virus until the host could generate a response against the new variant epitope. By that time, the virus could have already produced new antagonistic variants, and in this way, continually stay one step ahead of the immune response^{59,60}. In support of this model, a recent study showed that a cell line infected with a viable HIV mutant could inhibit the ability of a CTL line to lyse cells infected with wild-type virus⁶¹. Maybe HIV is exploiting this Achilles' heel of T cells, but further studies are needed to ascertain its role in the pathogenesis of HIV.

Treatment of autoimmunity. The ability of T cells to respond to altered ligands raises the possibility of a way to control specifically the activation of T cells with defined specificities, such as those involved in an autoimmune response. There have now been several reports on the use of APLs to inhibit experimental autoimmune encephalomyelitis *in vivo*^{62–65}. Furthermore, the demonstration that APLs can cause T cells to secrete unique patterns of cytokines also raises the possibility that APLs may be useful in forcing the immune system to deviate from an otherwise autoaggressive course^{66–69}.

The fact that the ability of T cells to interact with altered ligands has been retained by the immune system suggests that it is required for a competent immunological response. Therefore, Burke's contention that our antagonist is our helper must surely apply to the immune system. But, like Achilles, the developmental process that gives T cells their strength and versatility will also create a 'heel' that can be exploited by clever enemies such as viruses, and can give scientists a tool for solving problems. □

Gilbert J. Kersh and Paul M. Allen are at the Center for Immunology and the Department of Pathology, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, Missouri 63110, USA.

- Bartlett, J. *Familiar Quotations: A Collection of Passages, Phrases, and Proverbs Traced to their Sources in Ancient and Modern Literature* (Little Brown, Boston, 1901).
- Davis, M. M. & Bjorkman, P. J. *Nature* **334**, 395–402 (1988).
- Marrack, P. & Kappler, J. W. *Sci. Am.* **269**, 86–89 (1993).
- Samelson, L. E. & Klausner, R. D. *J. Biol. Chem.* **267**, 24913–24916 (1992).
- Chan, A. C., Desai, D. M. & Weiss, A. A. *Rev. Immun.* **12**, 555–592 (1994).
- Perlmutter, R. M. *et al.* *Rev. Immun.* **11**, 451–499 (1993).
- Chan, A. C., Iwashima, M., Turck, C. W. & Weiss, A. *Cell* **71**, 649–662 (1992).
- Pastor, M. I., Reif, K. & Cantrell, D. *Immun. Today* **16**, 159–164 (1995).
- Matsui, K. *et al.* *Science* **254**, 1788–1791 (1991).
- Weber, S. *et al.* *Nature* **356**, 793–796 (1992).
- Sykulev, Y. *et al.* *Immunity* **1**, 15–22 (1994).
- Corr, M. *et al.* *Science* **265**, 946–949 (1994).
- Bjorkman, P. J. *et al.* *Nature* **329**, 506–512 (1987).
- Madden, D. R., Gorga, J. C., Strominger, J. L. & Wiley, D. C. *Nature* **353**, 321–325 (1991).
- Fremont, D. H. *et al.* *Science* **257**, 919–927 (1992).
- Stern, L. J. *et al.* *Nature* **368**, 215–221 (1994).
- Bhardwaj, V., Kumar, V., Geysen, H. M. & Sercarz, E. E. *J. Immun.* **151**, 5000–5010 (1993).
- Evavold, B. D. *et al.* *Immunity* **2**, 655–663 (1995).
- Wucherpfennig, K. W. & Strominger, J. L. *Cell* **80**, 695–705 (1995).
- Vaitutti, S. *et al.* *Nature* **375**, 148–151 (1995).
- McKeithan, T. W. *Proc. natn. Acad. Sci. U.S.A.* **92**, 5042–5046 (1995).
- Evavold, B. D. & Allen, P. M. *Science* **252**, 1308–1310 (1991).
- Evavold, B. D., Sloan-Lancaster, J. & Allen, P. M. *Immun. Today* **14**, 602–609 (1993).
- De Magistris, M. T. *et al.* *Cell* **68**, 625–634 (1992).
- Jameson, S. C., Carbone, F. R. & Bevan, M. J. *J. exp. Med.* **177**, 1541–1550 (1993).
- Racioppi, L., Ronchese, F., Matis, L. A. & Germann, R. N. *J. exp. Med.* **177**, 1047–1060 (1993).
- Alexander, J. *et al.* *J. Immun.* **150**, 1–7 (1993).
- Evavold, B. D., Sloan-Lancaster, J., Hsu, B. L. & Allen, P. M. *J. Immun.* **150**, 3131–3140 (1993).
- Snook, K. *et al.* *J. Immun.* **151**, 6815–6821 (1993).
- Evavold, B. D., Sloan-Lancaster, J. & Allen, P. M. *Proc. natn. Acad. Sci. U.S.A.* **91**, 2300–2304 (1993).
- Cao, W. *et al.* *Nature* **378**, 295–398 (1995).
- Sloan-Lancaster, J., Evavold, B. D. & Allen, P. M. *Nature* **363**, 156–159 (1993).
- Sloan-Lancaster, J., Evavold, B. D. & Allen, P. M. *J. exp. Med.* **180**, 1195–1205 (1994).

- Jenkins, M. K. & Schwartz, R. H. *J. exp. Med.* **165**, 302–319 (1987).
- Schwartz, R. H. *Science* **248**, 1349–1356 (1990).
- Germain, R. N., Levine, E. H. & Madrenas, J. *The Immunologist* **3**, 113–121 (1995).
- Sette, A. *et al.* *Rev. Immun.* **12**, 413–431 (1994).
- Jameson, S. C. & Bevan, M. J. *Immunity* **2**, 1–11 (1995).
- Sloan-Lancaster, J., Shaw, A. S., Rothbard, J. B. & Allen, P. M. *Cell* **79**, 913–922 (1994).
- Madrenas, J. *et al.* *Science* **267**, 515–518 (1995).
- Weiss, A. & Littman, D. R. *Cell* **76**, 263–274 (1994).
- Sloan-Lancaster, J. & Allen, P. M. *Rev. Immun.* (in the press).
- Janeway, C. A. Jr *Immun. Today* **16**, 223–225 (1995).
- Vallittu, S. *et al.* *J. exp. Med.* **181**, 577–584 (1995).
- Matsui, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **91**, 12862–12866 (1994).
- Janeway, C. A. Jr *et al.* *Cold Spring Harb. Symp. q. Biol.* **54**, 657–666 (1989).
- Kisielow, P. & von Boehmer, H. *Adv. Immun.* **58**, 87–209 (1995).
- Robey, E. & Fowlkes, B. J. *Rev. Immun.* **12**, 675–705 (1994).
- Hogquist, K. A. *et al.* *Cell* **76**, 17–27 (1994).
- Szaboda, E. *et al.* *Science* **263**, 1615–1618 (1994).
- Ashton-Rickardt, P. G. *et al.* *Cell* **76**, 651–663 (1994).
- Ashton-Rickardt, P. G. & Tonegawa, S. *Immun. Today* **15**, 362–366 (1994).
- Jameson, S. C., Hogquist, K. A. & Bevan, M. J. *Nature* **369**, 750–752 (1994).
- Yagi, J. & Janeway, C. A. Jr *Int. Immun.* **2**, 83–89 (1990).
- Pircher, H. *et al.* *Nature* **351**, 482–485 (1991).
- Vasquez, N. J., Kane, L. P. & Hedrick, S. M. *Immunity* **1**, 45–56 (1994).
- Klenerman, P. *et al.* *Nature* **369**, 403–407 (1994).
- Bertoletti, A. *et al.* *Nature* **369**, 407–410 (1994).
- Allen, P. M. & Zinkernagel, R. M. *Nature* **369**, 355–356 (1994).
- Davenport, M. P. *Immun. Today* **16**, 432–436 (1995).
- Meier, U.-C. *et al.* *Science* **270**, 1360–1362 (1995).
- Smilek, D. E. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **88**, 9633–9637 (1991).
- Wauben, M. H. M. *et al.* *J. exp. Med.* **176**, 667–677 (1992).
- Franco, A. *et al.* *Eur. J. Immun.* **24**, 940–946 (1994).
- Kuchroo, V. K. *et al.* *J. Immun.* **153**, 3326–3336 (1994).
- Racke, M. K. *et al.* *J. exp. Med.* **180**, 1961–1966 (1994).
- Pfeiffer, C. *et al.* *J. exp. Med.* **181**, 1569–1574 (1995).
- Windhagen, A. *et al.* *Immunity* **2**, 373–380 (1995).
- Nicholson, L. B. *et al.* *Immunity* **3**, 397–405 (1995).

A neural basis for lexical retrieval

Hanna Damasio^{*†}, Thomas J. Grabowski^{*}, Daniel Tranel^{*}, Richard D. Hichwa[‡] & Antonio R. Damasio^{*†}

^{*} Department of Neurology, Division of Behavioral Neurology and Cognitive Neuroscience, and [‡] Positron Emission Tomography Imaging Center, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA

[†] The Salk Institute for Biological Studies, La Jolla, California 92186, USA

Two parallel studies using positron emission tomography, one conducted in neurological patients with brain lesions, the other in normal individuals, indicate that the normal process of retrieving words that denote concrete entities depends in part on multiple regions of the left cerebral hemisphere, located outside the classic language areas. Moreover, anatomically separable regions tend to process words for distinct kinds of items.

A CENTRAL question in the neurobiology of language concerns the neural structures that become active when the word that denotes a person or object is recalled, and is either silently verbalized or vocalized; that is, when an item from the lexicon of a given language is retrieved and explicitly represented in the mind. The traditional answer, based largely on more than a century of aphasia studies, invokes a set of left cerebral hemisphere structures around the Sylvian fissure, among which Broca and Wernicke areas figure prominently¹. This assumes that the classic language areas are activated directly by non-language areas which support the concept for which a pertinent word is being retrieved. Studies of neurological patients²⁻⁷ have led to an alternative answer, however. Although perisylvian structures (including early auditory and somatomotor cortices) are indeed involved in the transient reconstruction and explicit phonemic representation of word forms, additional neural sites mediate between those that support conceptual knowledge and the perisylvian structures, thus triggering and conducting the process of reconstruction⁸. We propose that there would not be a single mediational site for all words, but rather that there are separable regions within a large network which would preferentially assist with the processing of words denoting varied kinds of entities. Specifically, we considered that the retrieval of words denoting entities belonging to three distinct conceptual categories—unique persons, non-unique animals, and non-unique tools—depends on separable regions in higher-order cortices of the left temporal lobe. The two studies reported below were designed to test this hypothesis. In the first study of 127 subjects with focal brain lesions, we found a reliable relationship between category-related word-retrieval defects and neural sites within the left temporal lobe. In the second study, of 9 normal subjects who participated in a positron emission tomography (PET) word-retrieval experiment, we found differential activation of left temporal sites, comparable to those revealed by the lesion study.

Lexical retrieval in subjects with lesions

The lesion test of the hypothesis used a visual naming experiment in 127 adults with single and stable focal brain lesions. We used subjects with lesions throughout the telencephalon, allowing for sampling in both hemispheres, within and outside the temporal region.

The task required the naming of 327 visually presented items: (1) photographs of the faces of well-known people, to be named at unique (subordinate) level; (2) animals, and (3) tools, both of which were to be named at non-unique (basic object) level. The subject was asked to provide the specific and most adequate word denoting each entity. A response was scored as correct if it conformed to that of normal controls matched for age and

education. Responses were only scored as incorrect when the subject had appropriately recognized the item, so that scores reflected true word-retrieval ability (Table 1(a)). For example, if a subject failed to recognize the 'skunk' stimulus (as exemplified in the response "Some kind of animal; I don't know what . . . just an animal"), then that item was not included in the naming score. If the subject recognized the item (as exemplified in the response "Oh, that animal makes a terrible smell if you get too close to it; it is black and white, and gets squashed on the road by cars sometimes"), the item was included in the naming score, either as a correct response if the name was provided ('skunk'), or as a failure response if the name was not provided. Thus whenever a naming response was counted as incorrect, we were certain that the subject knew the item that they failed to name. We cannot claim that the subjects' concept retrieval was as normal as in the premorbid state, but we can affirm that the concept was retrieved specifically enough to provide a notion of what the stimulus was.

We tested two predictions. (1) The disruption of word retrieval for all three categories would be correlated with regions in left but not right hemisphere. (2) The disruption of word retrieval for each category would be consistently correlated with separable neural sites within left higher-order cortices of the temporal region, namely, temporal pole (TP) and inferotemporal (IT) sectors (Fig. 1a).

The subjects were first classified according to their task performance, which was normal in 97 subjects (47 with left-hemisphere damage, 50 with right) and abnormal in 30). All but one of the latter had damage in the left hemisphere, thus supporting the first prediction. The scores of brain-damaged subjects who performed abnormally in each of the three word categories were significantly lower ($P < 0.001$ in each case) than those of the brain-damaged subjects who performed normally (Table 1b).

In addition to isolated word-retrieval defects for unique persons, animals or tools, the instances of two combined defects always involved 'persons/animals' or 'animals/tools', but never 'persons/tools' (Table 1a). When the naming of persons and tools was impaired in the same individual, the naming of animals was impaired as well. The non-occurrence of instances of the 'persons/tools' combination is statistically significant ($P < 0.001$; Table 1a). The anatomical evidence discussed below indicates why this combination of defects is not possible on the basis of a single lesion.

Each subject's lesion was reconstructed in three dimensions⁹. The lesions of the 29 left-hemisphere subjects with abnormal performance were analysed using MAP-3, a technique which allows the determination of the volumetric overlap of lesions from multiple subjects (Fig. 1b). The overlaps revealed that: (1) abnormal retrieval of words for persons was correlated with

damage clustered in the left TP; (2) abnormal retrieval of words for animals was correlated with damage in left IT (mostly anterior); and (3) abnormal retrieval of words for tools correlated with damage in posterolateral IT, along with the junction of lateral temporo-occipito-parietal cortices (a region we have designated as 'posterior IT+'). None of the normal subjects had damage in the sites scrutinized by our hypotheses (TP or IT), which means that there was no instance of a 'false negative' result. Only three subjects with word-retrieval defects had lesions outside the sites described above (two predominantly in the left superior temporal gyrus, the other in the left thalamus). Thus the second prediction was also supported, qualified only by noting that impaired retrieval of words for tools was correlated with damage that extended into

the left inferior parietal cortex and the temporo-occipital junction. To assess the reliability of the findings, we conducted a test using anatomical placement of lesion as the independent variable. We compared, for each word category, the naming scores of three groups of subjects: (1) those with lesions in TP only; (2) those with lesions in posterior IT+ only; and (3) those with lesions centred in IT that extended, in some instances, into either TP or posterior IT+, but not into both). A multivariate analysis of variance (MANOVA) yielded highly significant results for persons ($F(2, 20) = 14.85$, $P < 0.001$) and tools ($F(2, 20) = 6.65$, $P < 0.01$), and a marginally significant result for animals ($F(2, 20) = 3.30$, $P = 0.058$). These results support the conclusion that the anatomical placement of lesion is a crucial factor deter-

TABLE 1 Results of word retrieval experiment

TABLE 1 Results of word retrieval experiment			
(a) Word retrieval defects			
Combinations of defect	Number of subjects impaired	Total number of subjects with defect in category	
For persons only	7	Persons = 13	
For animals only	5		
For tools only	7	Animals = 16	
For persons, animals, and tools	4		
For persons and animals	2	Tools = 16	
For animals and tools	5		
For persons and tools	0		
	Total: 30		
(b) Naming performances in brain-damaged subjects			
	Normal	Mean (s.d.)	t-test outcome*
Persons	n = 114 89.3 (4.9)	n = 13 49.6 (23.1)	t(125) = -6.17, P < 0.001†
Animals	n = 111 93.6 (3.0)	n = 16 66.6 (22.5)	t(125) = -4.79, P < 0.001
Tools	n = 111 95.0 (3.1)	n = 16 68.2 (23.5)	t(125) = -4.56, P < 0.001

a, Distribution of defects. In addition to isolated defects for persons, animals or tools, the instances of two combined defects always involved persons/animals or animals/tools, but never persons/tools. The non-occurrence of a persons/tools combination is not due to chance (Fisher exact probability test, $P = 0.0001$). b, Naming scores for brain-damaged subjects who were classified as either normal or abnormal for naming in each of the three categories. The differences are statistically significant for all three groups. We tested 127 brain-damaged subjects with lesions caused by cerebrovascular disease (106), herpes simplex encephalitis (5) or temporal lobectomy (16). All had IQs of ≥ 90 , had high-school education or higher, had been extensively characterized neuropsychologically and neuroanatomically, and had no difficulty with the attentive inspection of visual stimuli; 115 were right-handed, 6 were left-handed and 6 were ambidextrous. Normal controls ($n = 55$), drawn from another study⁷, were matched to brain-damaged subjects for age and education. (Sex differences in visual recognition and naming are generally small³⁶, so we did not analyse the data separately for each sex. The experimental and control groups contained similar proportions of men and women.) Unique stimuli were faces of famous people known to the subjects before their brain damage. Pictures were from the Iowa Famous Faces Test³⁷ and a modified version of the Boston Famous Faces Test³⁸; they were presented as black-and-white slides with all non-face background features deleted. Non-unique stimuli were line drawings³⁹ or photos⁷ of animals ($n = 90$) or tools ($n = 104$). Stimuli were shown in random order on a slide projector, without time limits. Responses were taped, transcribed and scored by raters who were blind to the experimental hypotheses. A naming response was correct if it was the same as that provided by normal controls. The response was only scored if the subjects demonstrated that they recognized the stimulus, either by naming it correctly or by providing a specific description of the entity (see text). In no case did we observe correct naming of a stimulus without subsequent recognition of the same stimulus. The naming score is the percentage of recognized items that were correctly named; this value is unaffected by the number of items that were not recognized, and thus distinguishes naming defects from recognition defects. We did, however, exclude from analysis any subjects who were unable to recognize at least 50% of items in each category; in most cases the recognition rate was much higher. Our scoring procedure ensures that the defects reported here pertain to word retrieval rather than to recognition impairments, even in subjects who showed defects in both naming and recognition. Brain-damaged subjects were classified as abnormal for naming in a particular category if their naming performance was > 2 s.d. below the mean for the normal controls.

* All t-tests were conducted with correction for unequal variances.

† Bonferroni-corrected P -values have been used.

FIG. 1 *a*, Regions investigated in the lesion study include TP, the left temporal pole, and IT, the inferotemporal region; stg indicates superior temporal gyrus. *b*, Results of MAP-3 analysis based on magnetic resonance (MR) or computed tomography (CT) scans processed for three-dimensional (3D) reconstruction in each subject with Brainvox⁹. The anatomical description of the lesion and of its placement relative to neuroanatomical landmarks was performed on-screen. Top, defective retrieval of words for persons; middle, defective retrieval of words for animals; bottom, defective retrieval of words for tools. One subject (right-handed) had a word-retrieval defect and a lesion in the right temporal region. All other subjects with word-retrieval defects ($n = 29$) had left-hemisphere lesions. The overlaps relative to the word-retrieval defects indicate three things. (1) Abnormal retrieval of words for persons correlated with damage clustered in left TP, trespassing minimally into IT. The maximal overlap included the lateral, inferior and mesial aspects of TP, and concerned both cortex and subcortical white matter. (2) Abnormal retrieval of words for animals correlated with damage in left IT; maximal overlap occurred in lateral and inferior IT regions. The anterior part of TP was not included in this overlap; the subcortical overlap was subjacent to the cortical damage and extended posteriorly, lateral to the temporal horn and the lower segment of the trigone. (3) Abnormal retrieval of words for tools correlated with damage in posterolateral IT, along with the junction of lateral temporo-occipito-parietal cortices (posterior IT+). The maximal overlap occurred at the posterior end of the middle temporal gyrus and the anteroinferior sector of the supra-marginal gyrus. The subcortical overlap was distinct from the one seen in (2) by being both posterior and superior to it. MAP-3 requires the transfer of each subject's lesion onto a normal 3D brain. MR images are obtained with an SPGR sequence of thin (1.5–1.7 mm) contiguous T₁-weighted coronal cuts. The normal brain is resliced, to match the slices of each subject's MR/CT and create a correspondence between these and the normal resliced brain. The contour of the lesion on each slice is then transposed onto the matched slices of the normal brain, taking into consideration the appropriate anatomical landmarks. For each lesion, the collection of contours constitutes an 'object'. The objects in any given group can intersect in space, yielding a maximal overlap relative to both surface damage and depth extension. The number of subjects contributing to the overlap is then determined and marked by the colour bar codes.

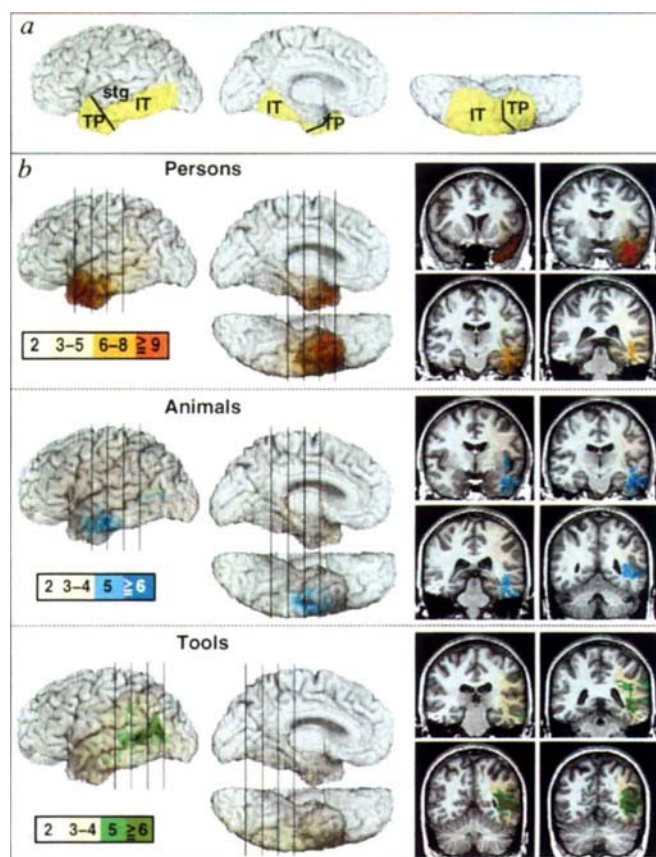
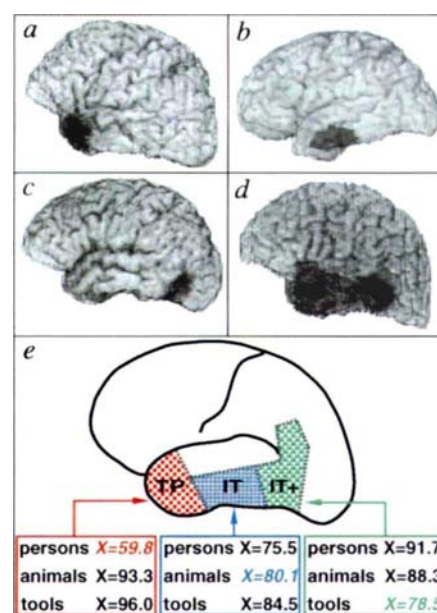


FIG. 2 *a–d*, 3D reconstructed brains of subjects with defects in word retrieval for single conceptual categories (*a–c*) and a defect in all three categories (*d*). The subjects had defective retrieval of words for *a*, persons only (the lesion was in the most anterior sector of left TP); *b*, animals only (lesion in the anterior sector of left IT overlapping minimally into the posterior part of left TP); *c*, tools only (lesion in the very posterior sector of IT and the most anterior part of the lateral occipital region (posterior IT+)); *d*, all three categories (lesion in all regions involved separately in the first three subjects, namely, the left TP, IT and posterior IT+). *e*, For each word category, the naming scores of three groups of subjects were calculated, with lesions in: TP only (red, $n = 6$); posterior IT+ only (green, $n = 6$); IT (blue, with outer borders overlapping in some instances into either TP or posterior IT+; $n = 11$). We did not include in this breakdown the 3 subjects whose lesions involve all three regions. For persons, the TP group had the lowest score ($X = 59.8$), followed by the IT group ($X = 75.5$) and the posterior IT+ group ($X = 91.7$). For animals, the IT group had the lowest score ($X = 80.1$), followed by the posterior IT+ group ($X = 88.3$) and the TP group ($X = 93.3$). For tools, the posterior IT+ group had the lowest score ($X = 78.5$), followed by the IT group ($X = 84.5$) and the TP group ($X = 96.0$). These differences were significant by MANOVA.



mining word-retrieval performance (Fig. 1). The close link between neuroanatomical structure and lexical category is also evident at individual subject level (Fig. 2).

Thus, impaired retrieval of words for entities was correlated with damage in higher-order cortices outside the classic language areas. Moreover, impairments in the three word categories were correlated in a consistent manner with separable neural sites. Two of these, TP and posterior IT+, are not even contiguous, do not

overlap cortically or subcortically, and are so distant as to make it virtually impossible for a single lesion to compromise them without also compromising the intervening region. This explains why a combined defect for persons and tools never occurred in our sample.

Lexical retrieval in normal subjects

To test our hypothesis with a radically different approach, we

TABLE 2 Summary of PET activation data

(a) Intensity thresholding					
	X	Y	Z	mm ³	t
Naming persons					
Left TP	-37	+3	-33	1,022	4.57
Left MTG	-56	-14	-9	93	4.11
Right TP	+42	0	-34	1,090	4.50
Naming animals					
Left IT	-37	-35	-15	3,339	5.43
Left TP	-30	0	-31	270	4.36
Naming tools					
Left IT	-52	-46	-12	2,636	5.36
Left IT	-31	-27	-19	838	5.29
Left TP	-29	-1	-33	626	4.82
(b) Spatial-extent thresholding					
	X	Y	Z	mm ³	p
Naming persons					
Left TP	-38	+4	-31	3,916	0.006
Right TP	+42	0	-33	3,269	0.009
Naming animals					
Left IT	-36	-31	-18	8,292	0.0006
Naming tools					
Left IT	-50	-50	-11	5,492	0.003
Left IT	-29	-28	-19	1,938	0.02
Left TP	-32	-1	-33	1,540	0.03
(c) Post-hoc analysis, spatial-extent thresholding					
	X	Y	Z	mm ³	p
Naming persons					
Left TP	-40	+5	-29	4,586	0.037
Left IFG/ACing	-22	+34	+17	36,232	<.0001
Mesial Parietal	-3	-61	+25	7,708	0.008
Naming animals					
Left IT	-35	-30	-16	10,575	0.002
Left IFG	-41	+22	+3	7,692	0.008
Right MSO	+5	-78	+21	4,476	0.039
Naming tools					
Left IT	-52	-50	-10	7,262	0.009
Left IFG	-45	+22	+9	16,244	0.002
Left ACing	-5	+35	+30	4,882	0.03

All regions of significant blood flow increase in TP/IT for the three naming tasks are shown, relative to the control task, using (a) the intensity-thresholding technique, and (b) the spatial extent-thresholding technique. These regions are characterized by the T_{88} coordinates⁴⁰ of their centres of mass, volume (mm³), maximal *t*-statistic (for intensity thresholding) and the corrected probability of type I error (for extent thresholding). (c) All regions of significant blood flow increase in TP/IT for the three naming tasks, relative to the control task, over the entire scanned intracerebral volume, analysed *post hoc* with spatial extent thresholding. (Although this exploratory analysis is included for sake of completeness, space does not allow discussion of the extratemporal foci, which were not the target of our hypotheses.) The naming tasks were referred to the same control task rather than directly to one another because we believe that lexical retrieval is mediated by a system of distributed functional units. When we predict activation in a certain region of this system for a given category, we expect that activation is weighted towards this component, but would also expect lower-level activation of other regions in the system. Direct comparison of the naming tasks to one another would be expected to be less sensitive to these regionalized effects, inasmuch as the image to be subtracted will also contain effects of the activation of the system⁴¹. When the naming tasks were directly compared, effects in the same locations were evident, but diminished in magnitude, as predicted. When naming non-unique entities (animals or tools) was subtracted from naming persons, a significant response remained in the right and left temporal poles (+46 +1 -27 corrected, $P = 0.003$; and -46 -4 -28, corrected $P = 0.03$, respectively) and in the lateral left middle temporal gyrus (-60 -19 -10, corrected $P = 0.01$). When naming animals was subtracted from naming tools, the activation in left posterolateral IT remained significant (-57 -54 0, corrected $P = 0.044$). When naming tools was subtracted from naming animals, the locus of activation seen in the main analysis for animals was diminished below the level of corrected statistical significance, but a local maximum with a *t*-score of 2.56 ($P = 0.006$ uncorrected) remained at -37 -40 -7, reflecting the relatively stronger activation of this portion of IT when naming animals. Thus a direct subtraction strategy gives results which have the same topography, with diminished but still significant standard scores. TP, temporal pole; IT, inferotemporal region; MTG, middle temporal gyrus; IFG, inferior frontal gyrus; ACing, anterior cingulate gyrus; MSO, mesial superior occipital region.

conducted a PET activation experiment using 9 normal right-handed adults (7 women and 2 men, aged 22–49). They were asked to name famous people (from photographs of faces), animals and tools (from a subset of the stimuli used in the lesion study) while their regional cerebral blood flow (rCBF) was measured. Following the results of the lesion study, we tested three predictions. (1) Retrieval of words denoting visually presented entities will activate left TP and IT regions. (2) Retrieval of words denoting entities from distinct conceptual categories will activate separate sectors of left TP/IT: left TP for persons; left IT for animals; and a sector posterior to that activated by naming animals (posterior IT+) for tools. (3) Naming of persons (but not of animals or tools) will also activate right TP/IT (we have evidence of the involvement of right TP/IT in the recognition of unique entities, and we did not expect naming of unique faces to occur without previous recognition of those faces). The rCBF in each word-retrieval condition was compared to rCBF generated by a single control task, in which subjects were shown unfamiliar faces, presented correct way up ('up') or upside down ('down'), and were asked to say 'up' or 'down'. Subjects therefore made a

vocal response but no naming was required. We chose unknown faces for the control task, because they do not evoke a name and yet depict real entities at least as complex as any of the stimuli in the naming tasks. Because all tasks were referred to the same control, any differences among the contrasts should be attributable to differences among the naming tasks themselves (Table 2).

Voxels (volume elements) corresponding to TP/IT were identified with co-registered magnetic resonance images of each subject's brain¹⁰ (Fig. 3). Regions of statistically significant increase in rCBF were identified in left TP/IT for each of the three naming tasks (Table 2), supporting the first prediction. The second prediction was also supported, because increases occurred in left ventrolateral TP but not in left IT when subjects named persons (Fig. 4 and Table 2). By contrast, naming both animals and tools activated left posterior IT and a restricted portion of the left TP, but with distinct patterns: activation for tool naming was seen in posterior middle and inferior temporal gyri, 12 mm posterior and 15 mm lateral to the region activated by naming animals, which was in the fourth and inferior temporal gyri (Table 2a and Fig. 4b). (We have shown that the 95% confidence radius associated with foci that are analysed with this general linear model is approximately 10 mm, at this sample size¹¹. These foci, separated by 17 mm in three dimensions, are distinguishable with confidence.)

As expected (from the third prediction), the person-naming task also activated right TP. The control task used unfamiliar rather than familiar faces, and thus did not subtract activity generated by the inevitable recognition of each person before naming. (These findings emphasize the difficulty of separating recognition and naming processes in normal subjects, as suggested by cognitive studies¹² and a previous PET study in which a task of categorizing persons by occupation¹³ activated TP bilaterally.)

We found activation in a small region of left TP (centred at Talairach T_{88} coordinates $-38, -8, -34$) for all word categories. This sector is less extensive, posterior and mesial to that activated by naming persons. It is also activated by a verb generation task¹¹, and may be the same sector as that identified in electrophysiological studies in which common nouns were used¹⁴. This sector may be related to lexical processing in general, rather than to specific categorical retrieval.

Generation of action words has been found to activate a portion of the left middle temporal gyrus^{11,15,16}. The area activated by naming tools in our study is 13–17 mm ventral to the reported coordinates of that area, and is probably functionally separate¹¹. (We have also noted the proximity of regions supporting retrieval of words for tools and retrieval of words for actions in the lesion results.)

Discussion

Our results indicate that the normal retrieval of words which denote concrete entities depends not just on classic language areas, but also on regions in higher-order association cortices. This has also been indicated by electrophysiological^{14,17} and functional imaging studies^{18,19}. Other evidence has indicated that impaired retrieval of words denoting actions is associated with damage to the left prefrontal/premotor region, and subjects with such lesions can retrieve words for entities normally^{20–22}. Thus there are dissociations relative to both type of word (words for entities versus words for actions) and anatomical locus (left temporal versus left frontal), which are also borne out by PET¹⁹ and electrophysiological evidence²³.

We suggest that the regions identified in our study play an intermediary or mediational role in lexical retrieval. For example, when the concept of a given tool is evoked (based on the activation of several regions which support pertinent conceptual knowledge and promote its explicit representation in sensorimotor terms), an intermediary region becomes active and promotes (in the appropriate sensorimotor structures) the explicit representation of phonemic knowledge pertaining to the word form which denotes the given tool. When a concept from another category is evoked,

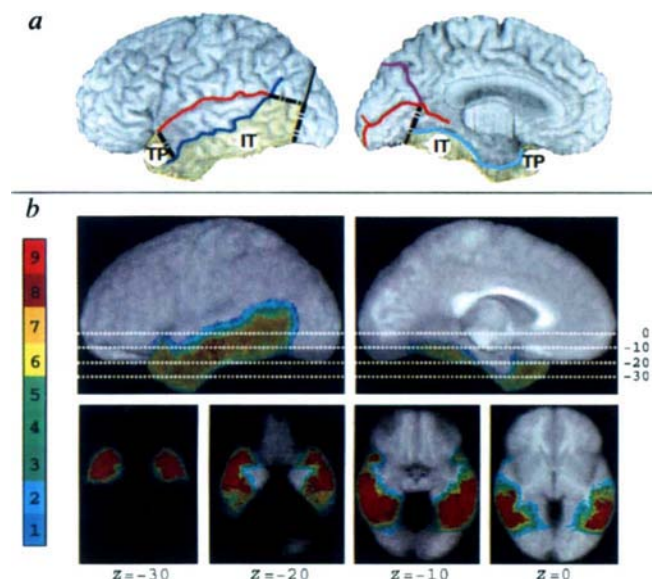


FIG. 3 a, A 3D reconstructed brain showing the limits of the TP/IT region. IT was defined as the middle, inferior and fourth temporal gyri, bounded superolaterally by the superior temporal sulcus (dark blue) and inferomedially by the collateral sulcus (light blue). The posterior boundary of the temporal lobe conformed to the definition in the atlas used⁴². Anteriorly, TP was separated from the superior temporal gyrus by a plane perpendicular to the longitudinal axis of the temporal lobe at the level of the anterior ascending ramus of the Sylvian fissure. All subjects had MR images reconstructed in three dimensions using Brainvox⁹ before the PET scanning session. The contiguous region-of-interest (TP/IT) was traced on each 3D brain reconstruction, in both hemispheres. b, The search volume used in the PET analysis was defined as all stereotactic voxels that corresponded to TP/IT in most subjects. It was determined by constructing the Talairach space for each subject^{11,40}. The TP/IT regions of interest were then converted into binary volumes, Talairach-transformed, and summed. Pixels with values reflecting the overlap of at least 5 subjects made up the search volume. Top, the lateral and mesial views of the Talairach-averaged brains of the 9 subjects in this study. Bottom, 4 axial slices obtained at the marked Z-levels. Colours identify the number of subjects who overlap at each pixel. The collection of 3D MR data enabled us to avoid several potential technical artifacts in this study. We eliminated extracerebral pixels from the data set and included in the search volume only those pixels corresponding to TP/IT in most subjects. We are confident that all of the regions of activation that we detected were either exclusively in TP/IT, or if they extended beyond its boundary, were centred within it.

that of a particular person for example, a different intermediary region is engaged. The process can operate in reverse to link word form information to conceptual knowledge. The intermediary regions do not contain in explicit form the names for all persons, animals or tools. We suggest, instead, that they hold knowledge about how to reconstruct a certain pattern (for example, the phonemic structure of a given word) in explicit form, within the appropriate sensorimotor structures. The intermediary role outlined here has a counterpart in the cognitive and linguistic proposals by Levelt²⁴ and Garrett²⁵, among others, which also involve the interposition of a processing step between concepts being evoked and word forms being made explicit.

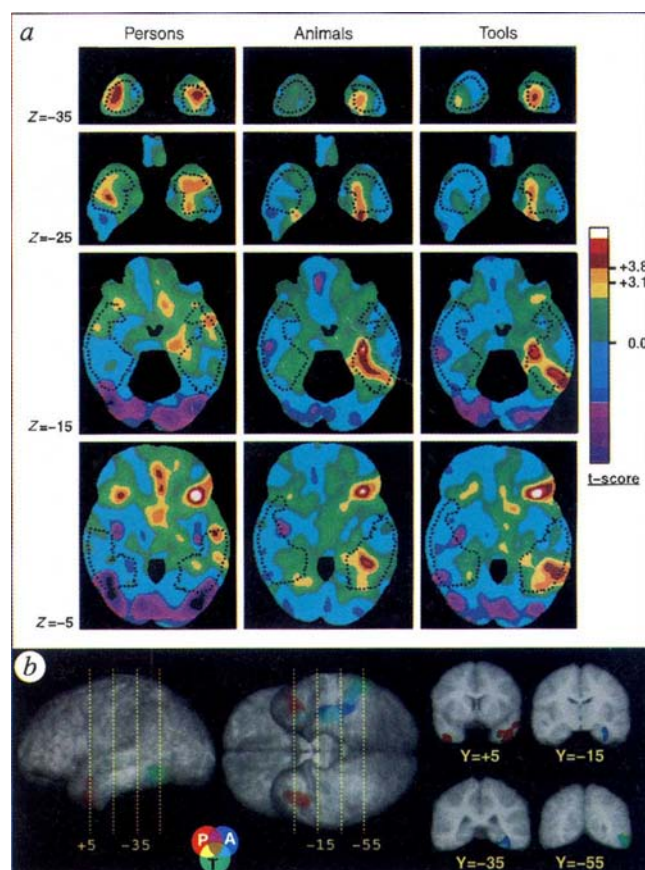
We suggest that there are two overriding reasons why naming of the different kinds of entities used in our experiments was correlated with different sites. The first involves the overall physical characteristics of the entity being named, which determine the sort of sensorimotor mapping generated during interactions between an organism and the entity, and which are a key to the neural mapping of the corresponding conceptual knowledge. For example, overall physical characteristics would govern the distinction among non-unique entities, such as manipulable tools and nonmanipulable animals. The second reason involves the fine physical characteristics and contextual linkages of an entity, which allow the mapping of unique items such as familiar persons or places. We presume that varied conceptual specification, which results from factors such as physical characteristics and contextual

complexity, is the driving force and 'principle' behind the differences in location of the lexical intermediary regions identified here. For instance, consider the multiple sensory channels (somatosensory, visual) and the hand motor patterns that are inherent in the conceptual description of a manipulable tool. These would result in the respective intermediary region being recruited near a sector of cortex that is capable of receiving such multiple sensory signals, and is close to regions involved in visual motion and hand motion processing. The intermediary region concerning names for persons would depend on both the finer physical specification and the contextual complexity necessary to define such unique items.

Our data do not address the issue of the possible microstructure of the intermediary regions, but we wish to specify our notion of those regions. We do not envisage them as rigid 'modules' or hard-edged 'centres', because we see their structure and operation as being acquired and modified by learning. An individual's learning experience of concepts of a similar kind (such as manipulable tools), and their corresponding words, leads to the recruitment, within the available neural architecture, of a critical set of spatially proximate microcircuits. We presume that the anatomical placement of the region within which the microcircuits are recruited for a certain range of items is not random. Rather, we suggest that it is the placement best suited to allow the most effective preferential interaction, by means of feedforward and feedback projections, between the regions of cerebral cortex which subtend conceptual

FIG. 4. a, The three columns of PET images demonstrate comparisons of adjusted mean activity in each of the naming conditions to the control task, using the *t*-statistic. The search volume is enclosed by the black dotted lines. The images are displayed without bottom thresholding. Red pixels are statistically significant after correction for multiple comparisons (see below). Distinct regions of activation are demonstrated in left TP/IT for each of the naming tasks (Table 2).

METHODS. Stimuli were presented in black-and-white on a video screen at a distance of 15 in. To produce uniform error rates of around 10%, face stimuli were presented every 2.5 s, tools every 1.8 s and animals every 1.5 s (stimuli for the control task were presented every 1.0 s). During the PET study, performance success was not significantly different among the naming tasks (non-response rates for naming persons, animals and tools were 17.0%, 14.8% and 11.7%). In all cases, stimuli were present for 25% of the presentation cycle. Stimuli were as described in Table 1 legend; to ensure that the faces used were indeed familiar, subjects inspected them 1–2 days before the PET scan, and indicated whether they recognized the person. At this time they were told not to name the person, nor was the name provided by the experimenter. Only familiar faces were used for the scan itself. MR data were used to orient the PET slices parallel to the longitudinal axis of the temporal lobe^{10,43}. PET data were acquired with a General Electric 4096 Plus whole-body tomograph, after intravenous bolus administration of 50 mCi of $H_2^{15}O$. Regional cerebral blood flow was estimated according to the autoradiographic method^{44,45}. MR and PET data were co-registered using PET-Brainvox^{10,43} and Automated Image Registration⁴⁶. The final calculated PET image resolution was $19 \times 19 \times 18$ mm. PET data were analysed with a pixelwise linear model^{41,47}. Global flow did not differ significantly across tasks. We compared adjusted mean activity in each of the three naming conditions to the control task using pixelwise *t*-statistics. The common intracerebral stereotactic volume was $1,106.3 \text{ cm}^3$. The search volume (bilateral TP/IT) was 123.4 cm^3 . The critical *t*-value (volumetric α 0.05, 58 degrees of freedom, 19 resels) was 3.82 (orange/red boundary on the colour scale)⁴⁸. We also analysed the *t*-fields using spatial extent rather than intensity thresholding. The *t*-fields were transformed to the normal distribution, a threshold of $Z = 3.0$ was applied (yellow/orange boundary in the images), and excursion regions of significant spatial extent were identified⁴⁹. Both thresholding techniques gave equivalent results (Table 2). b, Combined MR and PET data. Regions of activation for the three naming tasks, as determined by spatial extent thresholding (see above and Table 2(b)), are displayed superimposed on the average 3D MR volume of the participating subjects. The colour codes for regions activated by each task and for their areas of overlap are displayed on the Venn diagram (P, person naming; A, animal naming; T, tool naming). Four coronal slices through the volume are also displayed, with corresponding T_{88} Y-coordinates.



knowledge and those which can enact lexical knowledge. We suggest that distinct kinds of conceptual knowledge lead to the recruitment of distinct intermediary regions. (We have previously outlined some of the properties of the microcircuitry necessary for this mediational role; namely, convergence to and divergence from neuron ensembles which learn to associate certain patterns of feedforward signals from many sources with feedback responses to those same sources, and to other sites²⁶⁻²⁸.) We presume that the number of microcircuits recruited to operate as intermediaries for a certain range of concepts and words varies with the learning experience. Thus we expect normal individuals to develop, under similar conditions, a similar type of large-scale architecture, but we also predict ample individual variation of microcircuitry within each key region. We also expect that, at different times, the same individual will engage the same large-scale regions, but not necessarily the same microcircuitry within them.

We wondered whether, instead of having a solely mediational role, the regions identified by the available evidence might also

support conceptual knowledge. Naming responses in the lesion study were classified as defective only after the subject offered evidence of knowing what the stimulus was. Although we cannot be certain that conceptual evocation was entirely normal, we verified that the subjects retrieved knowledge specific to the concept. In addition, other studies that used the same stimuli in a concept-retrieval experiment have demonstrated that the regions most strongly associated with the three classes of concepts investigated here are remarkably different, with two of them being in the other hemisphere^{29,30}. Finally, we have preliminary and unpublished evidence that suggests that patients who fail to retrieve specific words in the experimental condition can still access the pertinent word-form knowledge in other conditions, such as phonemic cuing. Together with other findings³¹⁻³⁵, this suggests that the neural systems subserving conceptual and word-form knowledge for the same entity, are, in the very least, partly segregated; that the former probably influence the development of the latter; and that an interposed mediational system is a plausible notion. □

Received 31 October 1995; accepted 23 February 1996.

1. Damasio, A. R. *New Engl. J. Med.* **326**, 531-539 (1992).
2. Damasio, A. R., Damasio, H. & Van Hoesen, G. W. *Neurology* **32**, 331-341 (1982).
3. Warrington, E. K. & McCarthy, R. A. *Brain* **106**, 859-878 (1983).
4. Hart, J., Berndt, R. S. & Caramazza, A. *Nature* **316**, 439-440 (1985).
5. Goodglass, H., Wingfield, A., Hyde, M. R. & Theurkauf, J. C. *Cortex* **22**, 87-102 (1986).
6. Semenza, C. & Zettin, M. *Nature* **342**, 678-679 (1989).
7. Damasio, A. R., Damasio, H., Tranel, D. & Brandt, J. P. *Quant. Bio.* **55**, 1039-1047 (1990).
8. Damasio, A. R. & Damasio, H. *Scient. Am.* **267**, 89-95 (1992).
9. Damasio, H. & Frank, R. *Archs. Neurol.* **49**, 137-143 (1992).
10. Grabowski, T. J. et al. *Hum. Brain Mapp.* **2**, 123-133 (1995).
11. Grabowski, T. J. et al. *Hum. Brain Mapp.* (in the press).
12. Smith, E. E. & Medin, D. L. *Categories and Concepts* (Harvard Univ. Press, Cambridge, MA, 1981).
13. Sergent, J., Ohta, S. & MacDonald, B. *Brain* **115**, 15-36 (1992).
14. Nobre, A. C., Allison, T. & McCarthy, G. *Nature* **372**, 260-263 (1994).
15. Raichle, M. E. et al. *Cereb. Cort.* **4**, 8-26 (1994).
16. Martin, A., Haxby, J. V., Lalonde, F. M., Wiggs, C. L. & Ungerleider, L. G. *Science* **270**, 102-105 (1995).
17. Ojemann, G. A. *J. Neurosci.* **11**, 2281-2287 (1991).
18. Mazoyer, B. M. et al. *J. cogn. Neurosci.* **5**, 467-479 (1993).
19. Petersen, S. E., Fox, P. T., Posner, M. I., Mintun, M. & Raichle, M. E. *Nature* **331**, 585-589 (1988).
20. Damasio, A. R. & Tranel, D. *Proc. natn. Acad. Sci. U.S.A.* **90**, 4957-4960 (1993).
21. Daniele, A., Giustolisi, L., Silveri, M. C., Colosimo, C. & Gainotti, G. *Neuropsychologia* **32**, 1325-1341 (1994).
22. Hillis, A. E. & Caramazza, A. *J. cogn. Neurosci.* **7**, 396-407.
23. Dehaene, S. *NeuroReport* **6**, 2153-2157 (1995).
24. Levitt, W. J. M. *Cognition* **42**, 1-22 (1992).
25. Garrett, M. *Cognition* **42**, 143-180 (1992).
26. Damasio, A. R. *Cognition* **33**, 25-62 (1989).
27. Damasio, A. R. & Damasio, H. in *Large-Scale Neuronal Theories of the Brain* (ed. Koch, C.) 61-74 (MIT Press, Cambridge, MA, 1994).
28. Kosslyn, S. M. *Image and Brain* (MIT Press, Cambridge, MA, 1994).
29. Tranel, D., Damasio, H., Damasio, A. R. & Brandt, J. P. *Soc. Neurosci. Abstr.* **21**, 1497 (1995).
30. Perani, D. et al. *Soc. Neurosci. Abstr.* **21**, 1498 (1995).
31. Caramazza, A. & Hillis, A. *Nature* **349**, 788-790 (1991).
32. Hart, J. & Gordon, B. *Nature* **359**, 60-64 (1992).
33. Hillis, A. E. & Caramazza, A. *Brain* **114**, 2081-2094 (1991).
34. McCarthy, R. A. & Warrington, E. K. *Nature* **334**, 428-430 (1988).
35. Martin, A., Wiggs, C. L., Ungerleider, L. G. & Haxby, J. V. *Nature* **379**, 649-652 (1996).
36. McKenna, P. & Parry, R. *Neuropsychol. Rehab.* **4**, 255-281 (1994).
37. Tranel, D., Damasio, H. & Damasio, A. R. *J. cogn. Neurosci.* **7**, 425-432 (1995).
38. Albert, M. S., Butters, N. & Levin, J. A. *Archs Neurol.* **36**, 211-216 (1979).
39. Snodgrass, J. G. & Vanderwart, M. J. *exp. Psychol., hum. Learn. Mem.* **6**, 174-215 (1980).
40. Talairach, J. & Tournoux, P. *Co-planar Stereotaxic Atlas of the Human Brain* (Thieme Medical, New York, 1988).
41. Demonet, J. F. et al. *Brain* **115**, 1753-1768 (1992).
42. Ono, M., Kubik, S. & Abernathy, C. D. *Atlas of the Cerebral Sulci* (Thieme Medical, New York, 1990).
43. Damasio, H. et al. in *Quantification of Brain Function. Tracer Kinetics and Image Analysis in Brain PET* (eds Uemura, K., Lassen, N. A., Jones, T. & Kanno, I.) 465-473 (Elsevier, Amsterdam, 1993).
44. Herscovitch, P., Markham, J. & Raichle, M. E. *J. nucl. Med.* **24**, 782-789 (1983).
45. Hichwa, R. A. et al. in *Chemists' View of Imaging Centers* (ed. Emran, A. M.) 401-417 (Plenum, New York, 1995).
46. Woods, R. P., Mazziotta, J. C. & Cherry, S. R. *J. comput. assist. Tomogr.* **17**, 536-546 (1993).
47. Friston, K. J., Frith, C. D., Liddle, P. F. & Frackowiak, R. S. J. *J. Cereb. Blood Flow Metab.* **11**, 690-699 (1991).
48. Worsley, K. J. *Adv. Appl. Prob.* **26**, 13-42 (1994).
49. Friston, K. J., Worsley, K. J., Frackowiak, R. S. J., Mazziotta, J. C. & Evans, A. C. *Hum. Brain Mapp.* **1**, 210-220 (1994).

ACKNOWLEDGEMENTS. We thank R. Frank for collaboration in the development of MAP-3; J. Brandt, J. Spradling, K. Jones and D. Krutzfeldt for technical help; and L. Watkins and L. Ponto for assistance in the PET studies. This study was supported in part by grants from the National Institute for Neurological Diseases and Stroke and The Mathers Foundation.

CORRESPONDENCE AND MATERIALS. Requests to be addressed to A.R.D. (e-mail: antonio-damasio@uiowa.edu).

A one-plume model of martian mantle convection

Helmut Harder & Ulrich R. Christensen

Institut für Geophysik, Universität Göttingen, Herzberger Landstrasse 180, 37075 Göttingen, Germany

FOR at least the past two billion years, volcanism on Mars has been restricted to the Tharsis region¹. In addition, most tectonic activity on Mars², together with the long-wavelength topography and the non-hydrostatic gravity field³, are strongly correlated with Tharsis, implying a close connection with deep mantle processes. These observations have motivated suggestions⁴ that the thermal convection in the martian mantle is very different from that in the Earth's mantle, being dominated by a single large upwelling under Tharsis. Two-dimensional convection modelling has shown that the presence of an endothermic phase boundary in the lowermost mantle of a planet has a strong influence on convection, suppressing all but one or two upwellings⁵. Recent experiments⁶ indicate that such a boundary may indeed exist on Mars. Here we investigate the convective evolution of the martian mantle using a three-dimensional model which incorporates an endothermic phase transition close to the core–mantle boundary. We find that a single-plume pattern of convection gradually develops, and we show that this is consistent with the distribution of volcanism, the shape of the gravity field, and the gross tectonic stress pattern of Mars.

Previous three-dimensional models of convection in the martian mantle that ignored phase transitions showed a pattern with upwelling plumes⁷, but their number exceeded ten when the upper boundary was rigid rather than stress-free (as is appropriate for a one-plate planet) and when the mantle was mainly heated from within. Inclusion of the exothermic α – β and β – γ transitions in olivine and depth-dependent material properties into an axisymmetric convection model with a stress-free upper boundary also leads to several plumes⁸. For convection in the Earth, it has been demonstrated^{9–11} that the endothermic transition from the γ -phase to perovskite plus magnesio-wüstite has a strong influence on convection, which in the extreme case may shut off the mass flux across the phase boundary. Because of lower gravity this transition occurs much deeper in Mars than in the Earth, and because the core size is uncertain it is not clear if the transition pressure is reached at all in the martian mantle. The core radius can be constrained from the mass and the moment of inertia, together with cosmochemical inferences on the composition of core and mantle¹²; a rather small and dense core is required for the transition to perovskite to occur. The density of the (probably sulphur-rich) core has been assumed to be so low that the required large radius would prevent the occurrence of the perovskite phase. However, the recent discovery⁶ of a dense high-pressure high-temperature phase of FeS has led, for the preferred core composition with 14% sulphur, to a revised estimate of 2,000 km for the depth of the core–mantle boundary. This depth implies a perovskite layer of ~ 200 km thickness.

Recently, Weinstein⁵ used a two-dimensional model of convection in a cylindrical annulus to study the effects of an endothermic phase transition close to the annulus's inner boundary. He found that the phase change suppresses instabilities of the hot thermal boundary layer and leads, for pure bottom heating, to a single upwelling plume (or rather, to a sheet in the two-dimensional model). Two plumes were found in the more realistic case when internal heating was also present, and the core has a plausible size. Weinstein suggested that a single-plume pattern in the early Mars was the cause for the crustal dichotomy between the northern and southern hemispheres, but did not address the subsequent

evolution.

We have studied thermal convection in a three-dimensional spherical shell with a rigid upper boundary, representing the bottom of the lithosphere, and a stress-free lower boundary. All material parameters are constant and the adopted values (Table 1) are in a plausible range. We fix the temperature at the inner and outer boundaries and assume uniform internal heating. Its rate is roughly one-sixth of the chondritic value, which would imply a strong fractionation of heat-producing elements into the crust. The Rayleigh number, based on the temperature difference between inner and outer boundary, is 10^6 . In the model discussed here, the mean position of the divariant phase boundary is 120 km above the core–mantle boundary.

The calculation is started from a conductive temperature profile with an imposed almost dipolar perturbation. Initially a single overturn occurs. It leads to a temporarily stagnant mantle with an inverted thermal structure¹³ that could have been the post-accretional thermal state. After 1.75 billion years (1.75 Gyr) vigorous convection comprising several hot plumes begins (Fig. 1a). However, the weaker plumes die out rapidly and a pattern dominated by two plumes persists for a while (Fig. 1b). Eventually, only one plume survives (Fig. 1c, d). Its mean position remains fixed during the simulation, but its shape is modulated by additional boundary-layer instabilities. Monitoring the surface heat-flow shows that this plume is always among the three strongest during the first 1.5 Gyr after the onset of plume convection, that it clearly dominates after 1.5 Gyr, and that after 3.8 Gyr it is the only one. The downwelling flow consists predominantly of migrating sinking sheets. In the single-plume stage approximately 35% of the heat comes from the core, that is, heating is predominantly from within.

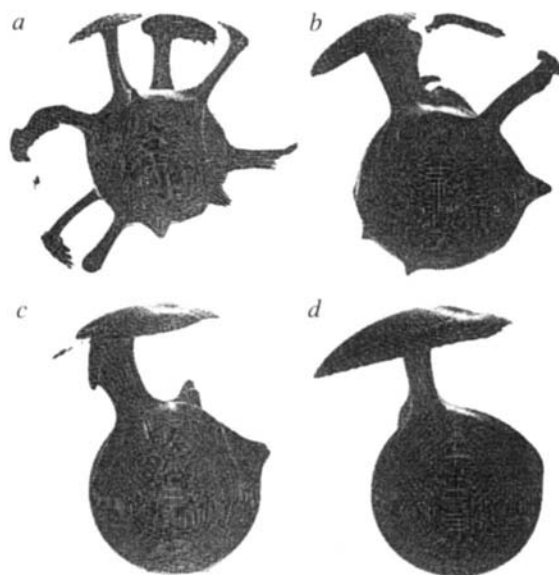


FIG. 1 Isosurfaces of temperature for a three-dimensional model of convection in Mars with a phase transition 120 km above the core–mantle boundary. The temperature contour at $0.53\Delta T$ (ΔT being the temperature difference between upper and lower boundary) highlights hot plumes. Snapshots are shown for 2.4 Gyr (a), 4.5 Gyr (b), 5.7 Gyr (c) and 7.8 Gyr (d) after the start of the calculation. The equations of incompressible viscous flow and of heat transport are solved by a semi-spectral technique similar to that used by Machetel *et al.*¹⁹. Spherical harmonics up to order and degree 96 are used, with 80 non-uniformly distributed radial grid levels. Deflections of the divariant phase boundary with a half-width of 30 km have been modelled as described by Christensen and Yuen⁹; latent-heat effects are ignored within the Boussinesq approximation used here.

TABLE 1 Model parameters

	Symbol	Value
Density	ρ	$3,500 \text{ kg m}^{-3}$
Gravitational acceleration	g	3.5 m s^{-2}
Thermal expansion coeff.	α	$2 \times 10^{-5} \text{ K}^{-1}$
Temperature difference	ΔT	500 K
Volumetric heating rate	Q	$3.25 \times 10^{-9} \text{ W m}^{-3}$
Mantle thickness	h	$2 \times 10^6 \text{ m}$
Core radius	r_c	$1.39 \times 10^6 \text{ m}$
Thermal diffusivity	κ	$10^{-6} \text{ m}^2 \text{ s}^{-1}$
Thermal conductivity	λ	$4 \text{ W m}^{-1} \text{ K}^{-1}$
Viscosity	η	10^{21} Pa s
Clapeyron slope	γ	$-2.6 \times 10^6 \text{ Pa K}^{-1}$
Phase density change	$\Delta \rho / \rho$	0.06
Rayleigh number		
–for basal heating	Ra_b	1×10^6
–for internal heating	Ra_Q	6.5×10^6

In simulations without a phase boundary, starting from the same initial state as before, 5–6 plumes persist. In one experiment we removed the phase boundary after the one-plume pattern had been established. Additional plumes appear again and a structure with five plumes of similar strength emerges (the pattern is similar to the one shown in Fig. 1a). An endothermic phase boundary tends to inhibit mass flux, but it does so more efficiently at short than at long wavelengths¹¹, which can explain our result. A phase boundary near the top of the hot thermal boundary layer suppresses small-scale instabilities in favour of a large strong upwelling. We found the relative mass flux across the phase boundary to be stronger in the late stage with a single plume compared to the early multi-plume stage. The evolution towards a single plume remains a robust feature for moderate variations of the Rayleigh number, the proportion of internal to bottom heating, the Clapeyron slope (which describes the dependence of phase transition pressure on temperature), or the location of the phase boundary above the core–mantle boundary (H.H., manuscript in preparation). However, the relative location of the two plumes

in the transient regime and the duration of the transient is strongly parameter-dependent.

To compare our model directly with observations, we calculated the resulting geoid anomaly and the pattern of surface stresses for a self-gravitating dynamic planet. For the stage shown in Fig. 1d, the predicted long-wavelength shape of the geoid is remarkably similar to the observed pattern (Fig. 2). But using the scaling parameters in Table 1, the amplitude is too small by almost an order of magnitude. The main source of the model geoid is the plume head in the upper mantle. A larger thermal expansivity at shallow depth compared to the assumed model value of $2 \times 10^{-5} \text{ K}^{-1}$, and the differential thinning of the lithosphere by the plume, could enhance the amplitude by a significant factor, but are unlikely to explain a misfit of a factor of ten. A larger effect can be expected from the additional buoyancy of the iron-poor residue left after melt extraction in the plume head¹⁴. High viscosity of the residue (due to the loss of volatiles¹⁵) could prevent it being swept away and could favour its accumulation in a layer of several hundred kilometres thickness beneath Tharsis, which would be necessary to explain the geoid amplitude¹⁶. The surface stresses (Fig. 3) reach their highest amplitude above the plume, with uniform extension in the centre and concentric extension prevailing in the periphery. This is consistent with the observed deformation pattern around Tharsis, which is dominated by rift structures radiating away from the centre of Tharsis².

Our results offer a framework for understanding the evolution of volcanic activity on Mars. Early in its history, volcanism on Mars was widespread¹, which may have to do with higher mantle temperatures but which probably also reflects the existence of numerous hot upwellings. Subsequently, volcanism was largely confined to the Tharsis and the Elysium provinces. Approximately 3.4–2.2 Gyr ago volcanism in Elysium became extinct, whereas the Tharsis volcanism continued up to almost recent times¹. This kind of evolution is mirrored in the development of the convective pattern in our model, which occur on a somewhat longer but roughly comparable timescale if we consider the start of vigorous convection as the initial state of Mars. Furthermore, the one

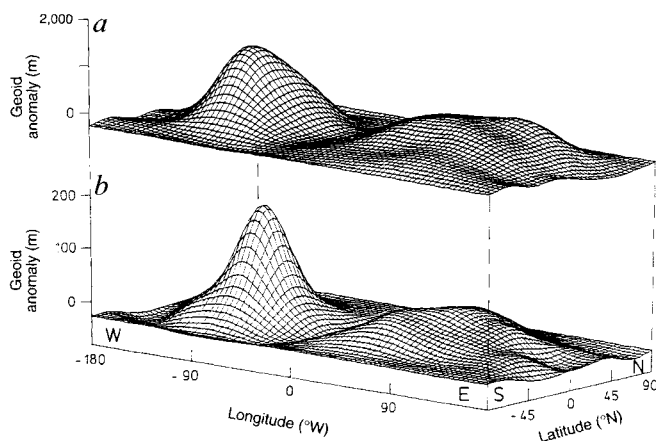


FIG. 2 Non-hydrostatic geoid anomalies of Mars truncated at harmonic degree and order eight. *a*, Observed^{3,20} with the hydrostatic contribution for a moment-of-inertia factor of 0.366. The peak is at 1,540 m. *b*, Calculated for the model with phase boundary, at 7.8 Gyr. The plume structure has been rotated to 110° W , 0° N in order to coincide with the centre of Tharsis. The maximum geoid height is 162 m. The dynamic geoid anomaly (including the effects of the induced surface and core topographies) is calculated for a self-gravitating planet with radially varying viscosity²¹. For the geoid calculation, we assume that a 150-km-thick viscous lithosphere overlies the isoviscous mantle, which represents the domain of our convection model. The lithospheric viscosity is 10^{26} Pa s above 100 km depth and decreases exponentially to 10^{21} Pa s at 150 km; the surface boundary condition is free slip.

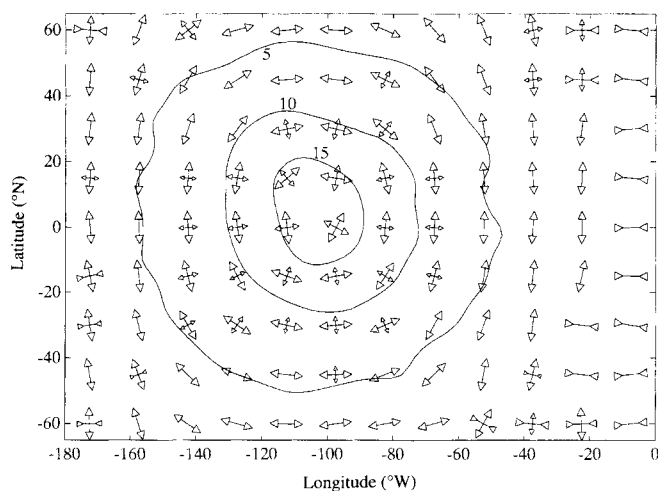


FIG. 3 Principal horizontal stress axes, and contours of stress magnitude (second invariant of deviatoric stress) in MPa, on the surface of a 150-km-thick viscous lithosphere overlying the plume structure in Fig. 1d. Viscosity parameters and rotation of the plume are the same as for the geoid calculation. Only the western (Tharsian) hemisphere is shown; on the eastern hemisphere, stresses are lower in magnitude and predominantly compressive. Arrowheads pointing outwards indicate extension, inwards compression. The length of the shorter of the crossing arrows indicates the magnitude of the smaller (in absolute value) principal stress in relation to the larger one, for which the arrow length is taken to be the same at all points. Where the secondary component is $< 50\%$ of the main component, no arrow is drawn.

surviving plume in the model remains nearly stationary during the whole evolution, which is consistent with the long duration of the Tharsis volcanism and tectonism, that started roughly 1 Gyr after the formation of Mars². Our model explains the pattern of the observed non-hydrostatic gravity field of Mars, but its large amplitude probably requires the accumulation of depleted low-density residue below Tharsis. Assuming that the lithosphere has negligible long-term elastic strength on the large length-scales, the calculated sign and orientation of surface stresses agrees with the prevailing deformation pattern around Tharsis (when the upward force of the plume is balanced by elastic flexure, the predicted stress pattern is inconsistent with observations¹⁷). Concerning stress and geoid, our results are close to the isostatic model of Sleep and Phillips¹⁶, which requires crustal thinning above Tharsis in order to fit simultaneously topography and gravity data. The high heat flow supplied by the plume could have been important in softening the lower crust sufficiently so that it was able to flow away from the Tharsis bulge¹⁸.

Mars is unique among the terrestrial planets because of its extreme monopolar distribution of younger volcanism, geoid anomalies, and to some extent tectonic deformation. Mars is also the only planet where the transition to perovskite can be expected to occur near the core-mantle boundary. Convection modelling establishes a link between these two observations,

because it shows that the deep phase boundary can enforce evolution towards a 'one-plume planet'. □

Received 10 November 1995; accepted 5 March 1996.

1. Neukum, G. & Hiller, K. *J. geophys. Res.* **86**, 3097–3121 (1981).
2. Banerdt, W. B., Golombek, M. P. & Tanaka, K. L. in *Mars* (eds Kieffer, H. H. et al.) 249–297 (Univ. Arizona Press, Tucson, 1992).
3. Esposito, P. B. et al. in *Mars* (eds Kieffer, H. H. et al.) 209–248 (Univ. Arizona Press, Tucson, 1992).
4. Phillips, R. J. & Ivins, E. R. *Phys. Earth planet. Inter.* **19**, 107–148 (1979).
5. Weinstein, S. J. *geophys. Res.* **100**, 11719–11728 (1995).
6. Fei, Y., Prewitt, C. T., Mao, H.-K. & Bertka, C. M. *Science* **268**, 1892–1894 (1995).
7. Schubert, G., Bercovic, D. & Glatzmeier, G. J. *geophys. Res.* **95**, 14105–14139 (1990).
8. Zhou, H., Breuer, D., Yuen, D. & Spohn, T. *Geophys. Res. Lett.* **22**, 1945–1948 (1995).
9. Christensen, U. R. & Yuen, D. A. *J. geophys. Res.* **90**, 10291–10300 (1985).
10. Machetel, P. & Weber, P. *Nature* **350**, 55–57 (1991).
11. Tackley, P. J., Stevenson, D. J., Glatzmeier, G. A. & Schubert, G. *J. geophys. Res.* **99**, 15877–15901 (1994).
12. Schubert, G. & Spohn, T. *J. geophys. Res.* **95**, 14095–14104 (1990).
13. Boss, A. P., Angevine, C. L. & Sacks, I. S. *Phys. Earth planet. Inter.* **36**, 328–336 (1984).
14. Phillips, R. J., Sleep, N. H. & Banerdt, W. B. *J. geophys. Res.* **95**, 5089–5100 (1990).
15. Karato, S. *Nature* **319**, 309–310 (1986).
16. Sleep, N. H. & Phillips, R. J. *J. geophys. Res.* **90**, 4469–4490 (1985).
17. Banerdt, W. B., Phillips, R. J., Sleep, N. H. & Saunders, R. S. *J. geophys. Res.* **87**, 9723–9733 (1982).
18. Schmeling, H. & Marquart, G. *Geophys. Res. Lett.* **17**, 2417–2420 (1990).
19. Machetel, P., Rabinowicz, M. & Bernadet, P. *Geophys. astrophys. Fluid Dyn.* **37**, 57–84 (1986).
20. Balmino, G., Moynot, B. & Valès, N. *J. geophys. Res.* **87**, 9735–9746 (1982).
21. Zhang, S. & Christensen, U. R. *Geophys. J. Int.* **114**, 531–547 (1993).

ACKNOWLEDGEMENTS. We thank L. Fleitout for a program to calculate the geoid. This work was supported by the Deutsche Forschungsgemeinschaft.

Three-dimensional electrical impedance tomography

P. Metherall, D. C. Barber, R. H. Smallwood & B. H. Brown

Department of Medical Physics and Clinical Engineering,
University of Sheffield, Royal Hallamshire Hospital, Glossop Road,
Sheffield S10 2JF, UK

THE electrical resistivity of mammalian tissues varies widely^{1–5} and is correlated with physiological function^{6–8}. Electrical impedance tomography (EIT) can be used to probe such variations *in vivo*, and offers a non-invasive means of imaging the internal conductivity distribution of the human body^{9–11}. But the computational complexity of EIT has severe practical limitations, and previous work has been restricted to considering image reconstruction as an essentially two-dimensional problem^{10,12}. This simplification can limit significantly the imaging capabilities of EIT, as the electric currents used to determine the conductivity variations will not in general be confined to a two-dimensional plane¹³. A few studies have attempted three-dimensional EIT image reconstruction^{14,15}, but have not yet succeeded in generating images of a quality suitable for clinical applications. Here we report the development of a three-dimensional EIT system with greatly improved imaging capabilities, which combines our 64-electrode data-collection apparatus¹⁶ with customized matrix inversion techniques. Our results demonstrate the practical potential of EIT for clinical applications, such as lung or brain imaging and diagnostic screening⁸.

Our methodology of two-dimensional (2D) EIT has been described in detail previously^{10,12,17}. Electrodes are positioned with equal spacing around the body to be imaged, thus defining a plane through the object. Voltage profiles are collected for all drive and receive electrode-pair combinations, and images are reconstructed as though the data were from a 2D object. In practice objects are three-dimensional (3D) and Fig. 1 shows 2D images obtained from such an object. These demonstrate that there are significant contributions to the image from off-plane

conductivity changes. This means that, unlike 3D X-ray images which can be constructed from a set of independent 2D images, for 3D EIT it is necessary to reconstruct images from data collected over the entire surface of the object volume¹².

In this work we have taken a sample of the boundary surface using 64 electrodes placed around the object (32 independent current drive and 32 voltage receive channels), Fig. 2. Current is driven between pairs of adjacent drive electrodes. For each drive pair, a set of voltage measurements are recorded from receive electrode pairs. Three data collection strategies have been studied to investigate the performance of image reconstruction with different boundary voltage data set sizes. These sequences were derived from knowledge of limitations in the data collection instrumentation¹⁶ and are shown in Fig. 2.

In order to generate the reconstruction algorithm, we have split the volume of interest into 4,608 tetrahedral elements. A sensitivity matrix, based on the theorem by Geselowitz^{18,19}, can be derived which relates the magnitude of small changes in conductivity, $\Delta c = c - c_{ref}$, and the resulting changes in potential differences $\Delta g = g - g_{ref}$, on the boundary of the object, by

$$\Delta g = S \Delta c \quad (1)$$

Although the sensitivity matrix S changes with the conductivity distribution and is therefore nonlinear, for small changes in c this can be ignored¹². We have argued elsewhere²⁰ that it is preferable to replace the perturbed data Δg by a set of potential differences normalized to the reference vector g_{ref} and in this case equation (1) becomes

$$\Delta g_n = F \Delta c_n \quad (2)$$

where Δg_n is the normalized change in boundary voltages and Δc_n is the normalized change in conductivity.

Matrix F is now the normalized sensitivity matrix in which the coefficient corresponding to a particular drive/receive pair and tetrahedral element is normalized by the sum of coefficients for the same drive/receive pair over all the elements. Although F is no better conditioned than S , it is less dependent on the requirements of a circular boundary shape and accurate electrode positioning^{12,20}. This is essential for clinical imaging applications, where a subject's profile does not match the model used to generate the sensitivity matrix and accurate placement of electrodes is difficult.

Inverting the above relationship (equation (2)) allows us to

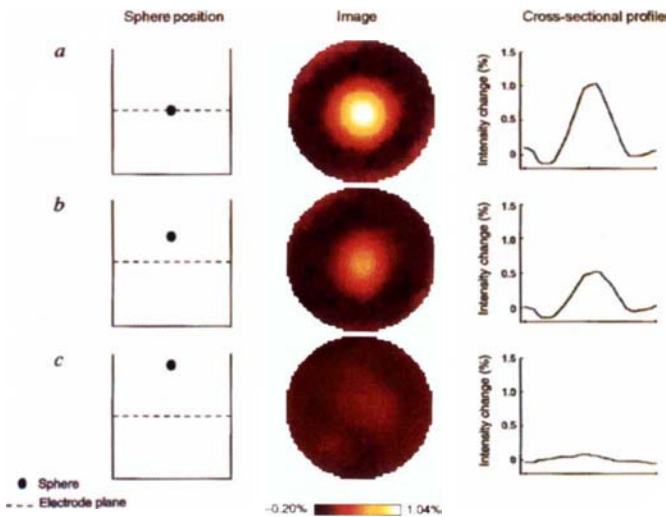


FIG. 1 Images showing how off-plane conductivity changes affect the reconstructed image in 2D EIT. As the conductivity change is moved further away from the electrode plane, its intensity in the image plane falls. But changes at distances equal to the tank (phantom) radius can still be resolved in the image plane. Radially offset changes show a similar effect, but with the further complication that off-plane changes are also shifted towards the centre of the image¹³. All three images are displayed using the colour scale shown. The maximum values of conductivity change, expressed as a percentage, are given.

METHODS. The images were produced using a 16-electrode system with interleaved drive and receive electrodes¹⁶. Current is driven through a pair of electrodes into the body and potential differences are measured between the other electrode pairs. The current drive pair is then rotated and another set of voltage measurements is collected. The phantom, of diameter 230 mm, was filled with saline solution (conductivity $\sigma = 4 \text{ mS cm}^{-1}$) and a reference (g_{ref}) voltage profile was collected. A small electrically insulating sphere of diameter 20 mm was then positioned as follows: *a*, in the electrode plane; *b*, at a distance of half the phantom radius from electrode plane; and *c*, at a distance of the phantom radius from electrode plane. Data sets (g) were recorded for each position, and the boundary voltage profile is then given by $\Delta g_n = (g - g_{\text{ref}})/g_{\text{ref}}$.

reconstruct the internal change in conductivity, Δc_n , from measured boundary data. Sensitivity coefficients, S , are formed for each tetrahedral element and for all drive/receive electrode-pair combinations. $\nabla \phi_d$ is the potential gradient that is generated within a tetrahedral element (e) when a unit current is driven between the drive pair electrodes. $\nabla \phi_r$ is the potential gradient which would be generated in the same element if unit current were driven through the receive pair. The sensitivity coefficient for each element and electrode pair combination is given by

$$S_{d,r,e} = - \int_e \nabla \phi_d \cdot \nabla \phi_r dV \quad (3)$$

where the integral is over the elemental volume. As in the 2D case, we calculate the sensitivity matrix assuming that the conductivity distribution is uniform^{12,19}. A full solution of the potential field ϕ is difficult¹⁰, so in this preliminary work we additionally assume the potential at a point in the object volume is given by²¹ $\phi = (r_1)^{-1} - (r_2)^{-1}$ where r_1 and r_2 are the distances of the point to the two electrodes forming the pair. The next step is to find the inverse matrix $\mathbf{F}^{-1}$. Considering the largest data set, the sensitivity

matrix will have 3,136 by 4,608 elements, and pseudo-inversion of this large rectangular matrix poses a formidable challenge. This can be achieved, however, by utilising the Moore–Penrose pseudo-inverse²² so that the reconstruction algorithm becomes $\Delta c_n = \mathbf{F}^+ \mathbf{Q}^\dagger \Delta g_n$ where $\mathbf{Q}$ is $(\mathbf{F}^T)^{-1}$. (Here t denotes the matrix transpose and $\dagger$ the pseudo-inverse.) Inversion of $\mathbf{Q}$ is still difficult although it is smaller than $\mathbf{F}$ and is a square matrix. If we collect the data from the cylindrical object in a strictly rotationally symmetric order, matrix $\mathbf{Q}$ will have the form:

$$\mathbf{Q} = \begin{bmatrix} Q_1 & Q_2 & Q_3 & Q_4 & Q_5 & Q_6 & Q_7 & Q_8 \\ Q_8 & Q_1 & Q_2 & Q_3 & Q_4 & Q_5 & Q_6 & Q_7 \\ Q_7 & Q_8 & Q_1 & Q_2 & Q_3 & Q_4 & Q_5 & Q_6 \\ Q_6 & Q_7 & Q_8 & Q_1 & Q_2 & Q_3 & Q_4 & Q_5 \\ Q_5 & Q_6 & Q_7 & Q_8 & Q_1 & Q_2 & Q_3 & Q_4 \\ Q_4 & Q_5 & Q_6 & Q_7 & Q_8 & Q_1 & Q_2 & Q_3 \\ Q_3 & Q_4 & Q_5 & Q_6 & Q_7 & Q_8 & Q_1 & Q_2 \\ Q_2 & Q_3 & Q_4 & Q_5 & Q_6 & Q_7 & Q_8 & Q_1 \end{bmatrix} \quad (4)$$

FIG. 2 Three-dimensional mesh, electrode position and pair configuration. To generate the reconstruction algorithm, a model representing the object volume is divided into 4,608 tetrahedral elements. These are configured as eight planes of 576 elements (*a*). On nodes at the surface of the cylinder, the electrode positions are shown. This is based on a 64-electrode data collection system with 32 independent current drive and voltage measurement (receive) electrodes. They are arranged in four planes of 16, with an interleaved configuration.

METHODS. Three data collection sequences were studied with different data set sizes. The first (*c*) includes 56 drive and 56 receive pairs, formed between electrodes in the same plane and between adjacent planes ($\Delta g_n = 3,136$ elements); the second (*d*) has only 32 in-plane receive pairs ($\Delta g_n = 1,792$ elements) and the third (*e*) has 32 drive and 32 receive pairs ($\Delta g_n = 1,024$ elements). The ordering of the data collection sequence is configured so that we have rotational symmetry. Current is first driven sequentially between pairs on all planes corresponding to drive segment 1, with a full set of voltage measurements collected for each drive pair. Current is then driven between pairs in segment 2, but the order of voltage measurements is shifted so that the geometrical relationship between the receive pairs and the drive pairs is identical for each segment (*b*).

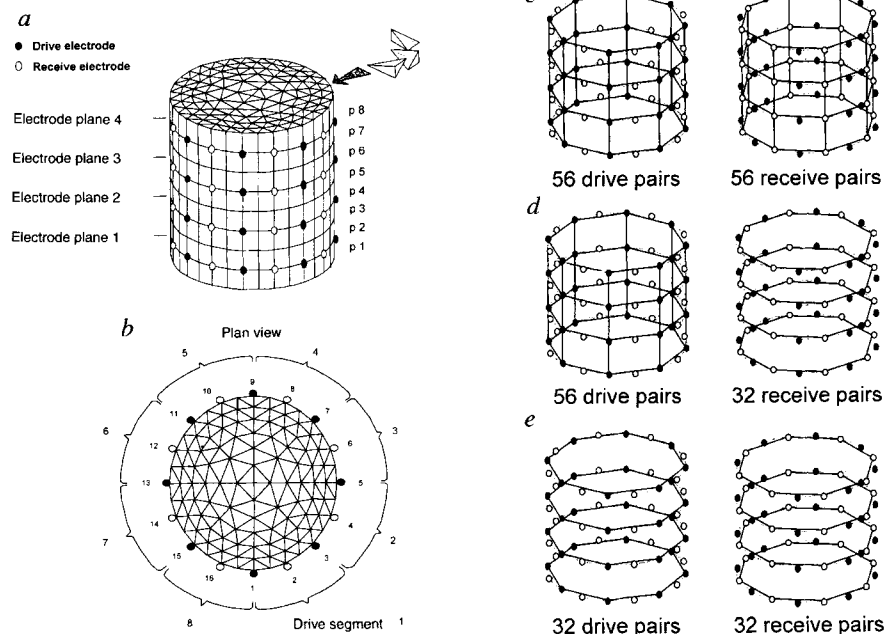
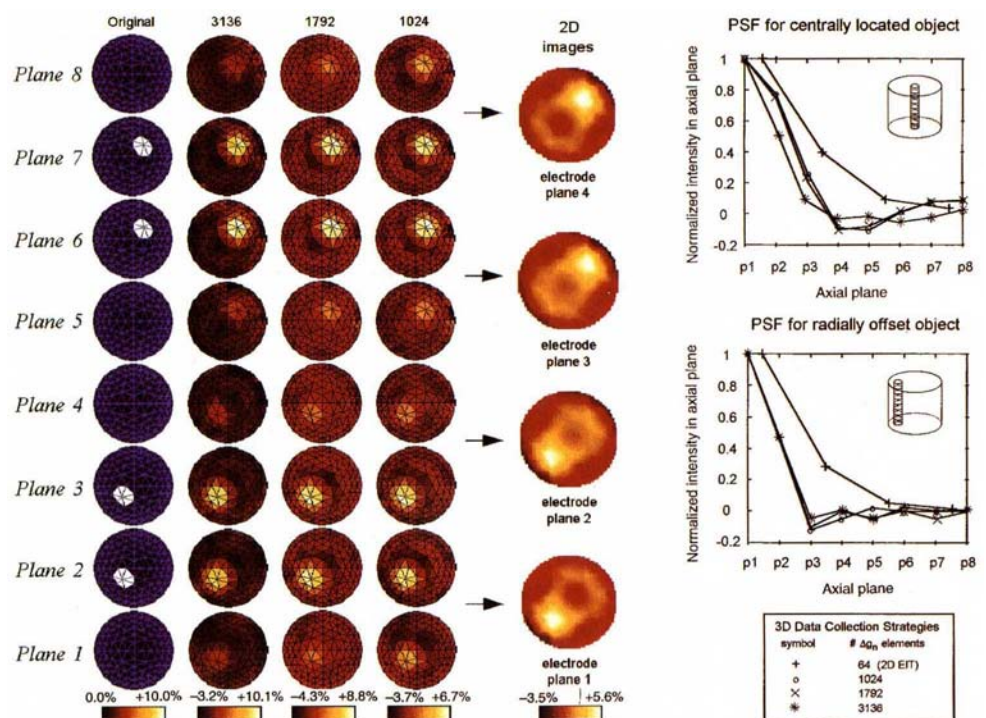


FIG. 3 Image sets for each data collection configuration are shown for comparison against the original conductivity change distribution from which the simulated boundary data set is calculated. This distribution represents two volumes of conductivity change positioned at different heights within the model. Each block spans two mesh planes. 2D images that correspond to the four separate electrode planes are also shown. These indicate increased spread into neighbouring planes, thus demonstrating the resolution improvement gained with 3D imaging. This is quantified in the point spread function (PSF) which indicates the performance between the different methods (increased PSF gradient corresponds to an improvement in axial resolution). The radially offset PSF is measured at a distance of two-thirds the simulated phantom radius from the central axis, and shows that image resolution is not uniform. METHODS. To display the internal conductivity changes, the cylinder (see Fig. 2a) has been split into its eight constituent planes and a plan view of each is given. A triangle in the figure thus represents a wedge containing three tetrahedral elements. The original image is generated by defining the conductivity change $\Delta\sigma_n$ in the required elements to 10% (white). All other elements (black) have no associated change. A simulated voltage profile is generated by multiplying the original $\Delta\sigma_n$ by the sensitivity matrix, equation (2). This is then reconstructed to give the images shown. Image sets are displayed using the colour scales given below each, with the



maximum percentage conductivity change. The PSF is generated by changing the conductivity of the element block shown in the lowermost plane (p1) only. Corresponding element intensities are measured for all eight mesh planes (p1–p8) in the reconstructed image and normalized to the first plane value.

Sub-matrix $\mathbf{Q}_k$ corresponds to all the data collected with current driven between electrode pairs in the k th drive segment (Fig. 2). To obtain this rotational symmetric format, the order of the receive pair measurements with respect to the drive pair is such that the geometry is identical for each drive segment apart from the required rotational shift. If, for example, the first receive pair for segment 1 drive pairs is between electrodes 2 and 4, the first receive pair for drive segment 2 will be between 4 and 6 (Fig. 2). The circular symmetric matrix $\mathbf{Q}$ can be transformed to a block diagonal matrix $\mathbf{D}$ which has the form $\mathbf{D}_{i,j} = \mathbf{D}_i \delta_{i,j}$ ($i, j = 1, 2, \dots, 8$), by taking the fast Fourier transform (FFT). If matrix $\mathbf{F}$ is order (m by n), the resulting $\mathbf{D}_i$ sub-matrices will be order ($m/8$ by $m/8$), as we have eight orders of rotational symmetry. These are pseudo-inverted independently using a method such as truncated singular value decomposition^{23,24}. The inverse $(\mathbf{F}\mathbf{F}^\dagger)^\dagger$ matrix is then formed when the inverse FFT is taken of the recombined inverted sub-matrices.

To compare the performance of the three data collection configurations, computer simulations have been carried out. Given a known distribution of conductivity change we can generate a simulated voltage profile, $\Delta\sigma_n$, using equation (2). We can then reconstruct back to observe how accurate the reconstructed image is compared to the original. In addition to the 3D models, we have also simulated the equivalent set of 2D images that would be produced from each of the four electrode planes. Figure 3 shows image sets for the 2D and 3D simulations. These demonstrate the improvement gained in axial resolution with 3D imaging. For the 2D case, we can see a similar result as shown in Fig. 1 where the effects of 3D $\Delta\sigma_n$ changes propagate into neighbouring planes. This phenomenon is reduced considerably for the 3D reconstructions and can be quantified by measuring the

response of a point object source in image space along an axially oriented axis.

Ideally a point object source will result in a corresponding point in the image, but inevitably some blurring will occur. This distribution is termed a point spread function (PSF) and Fig. 3 shows the PSF for each of the reconstruction methods in a central and radially offset position. As expected, the largest data set ($\Delta\sigma_n = 3,136$ measurements) gives the best response with the intensity in neighbouring planes reducing most rapidly. There appears only a marginal improvement between the 1,792 and 1,024 measurement data sets, suggesting that there is little useful information in the additional drive plane measurements. Because these are all made with drive and receive pairs in orthogonal planes, the signals produced have small values. The PSF also suggests that the axial resolution is improved at radially offset positions.

Using the 1,024-element data set configuration, data has been collected from a saline-filled tank of the same proportions as the modelled cylinder of Fig. 2. Electrically insulating objects were positioned within the phantom; the images collected are shown in Fig. 4. We also show for comparison a computer simulation of a similar 3D distribution. The real images are of lower quality than the simulated versions owing to the introduction of measurement noise by the data collection system and the use of an approximate potential field model in the sensitivity matrix calculation.

Although the spatial resolution of 3D EIT (about 10% of image diameter in the cross-sectional plane and 12.5% in the axial plane) is worse than that of other imaging techniques such as magnetic resonance imaging and X-ray computed tomography, it does have several distinct advantages over existing medical imaging methods. These include safety, portability, long-term monitoring, cost, and the inherent ability to image physiological function. We are

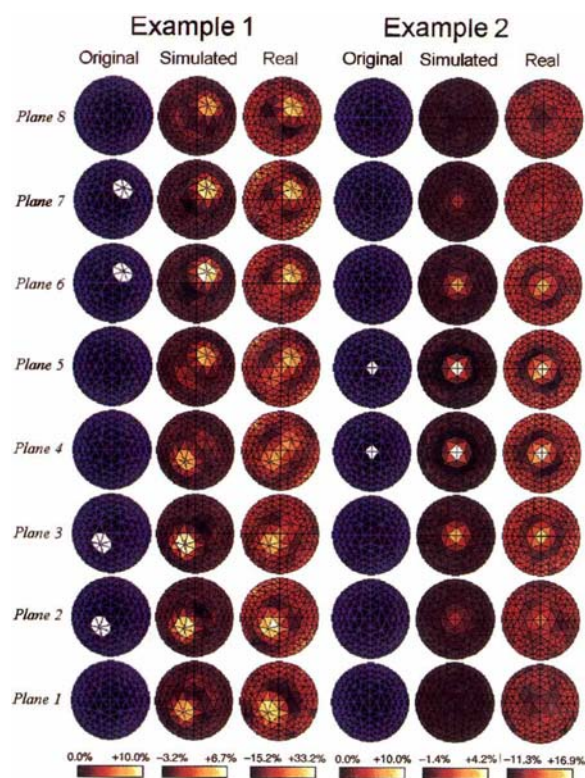


FIG. 4 Comparison of simulated and real images. The original images, of change in conductivity $\Delta\sigma_n$, give a representation of nylon cylindrical blocks (diameter 50 mm, length 60 mm) positioned in a saline-filled ($\sigma = 4 \text{ mS cm}^{-1}$) phantom. Example 1 corresponds to two blocks located at different heights and radial positions within the phantom. Example 2 corresponds to a single block positioned in the centre of the phantom. Simulated 3D images based on a 1,024 data set give a close representation of the original conductivity distribution with only limited $\Delta\sigma_n$ changes being propagated into adjacent planes. Real images, reconstructed from data collected from the phantom, are less well defined but the nylon blocks can still be identified.

METHODS. Simulated images are as described in Fig. 3. Real data were collected using a phantom with the same geometrical proportions as the 3D mesh of Fig. 2, using the Sheffield MK3b Electrical Impedance Tomographic Spectroscopy (EITS)¹⁶. This 64-electrode system applies current at eight frequencies over the range 9.6 kHz to 1.2 MHz, and acquires a complete data set in 60 ms. A reference data set was collected from a saline-filled phantom with uniform conductivity. Nylon blocks were then submerged at positions represented by the simulated forward $\Delta\sigma_n$ image, and another voltage measurement data set was collected. These are normalized to form the boundary data set Δg_n and reconstructed. Images are displayed with colour scales showing the maximum values of percentage conductivity change.

currently implementing a clinical trial to investigate the feasibility of using 3D EIT to detect pulmonary emboli. If the trial is successful, 3D EIT will provide an important alternative to the established radionuclide imaging technique. □

Received 10 October 1995; accepted 23 February 1996.

- Geddes, L. A. & Baker, L. E. *Med. Biol. Engng* **5**, 271–293 (1967).
- Duck, F. A. *Physical Properties of Tissue* 167–223 (Academic, London, 1990).
- Stoy, R. D., Foster, K. R. & Schwan, H. P. *Phys. Med. Biol.* **27**, 501–513 (1982).
- Pethig, R. *Clin. Phys. Physiol. Meas.* **A8**, 5–12 (1987). (see note below)
- McAdams, E. T. & Jossinet, J. *Physiol. Meas.* **A16**, A1–A14 (1995).
- Dawids, S. G. *Clin. Phys. Physiol. Meas.* **A8**, 175–180 (1987).
- Dijkstra, A. M. et al. *J. med. Engng Technol.* **17**, 89–98 (1993).
- Holder, D. S. & Brown, B. H. in *Clinical and Physiological Applications of Electrical Impedance Tomography* (ed. Holder, D. S.) 47–60 (University College London Press, London, 1993).
- Barber, D. C., Brown, B. H. & Freeston, I. L. *Electron. Lett.* **19**, 933–935 (1983).
- Barber, D. C. & Brown, B. H. *J. Phys. E: Sci. Instrum.* **17**, 723–733 (1984).
- Barber, D. C. in *Clinical and Physiological Applications of Electrical Impedance Tomography* (ed. Holder, D. S.) 47–60 (University College London Press, London, 1993).
- Barber, D. C. & Brown, B. H. in *Inverse Problems in Partial Differential Equations* (eds. Colton, D., Ewing, R., Rundell, W.) 151–164 (Soc. for Industrial and Applied Mathematics, Philadelphia, 1990).

- Rabbani, K. S. & Kabir, A. M. B. H. *Clin. Phys. Physiol. Meas.* **12**, 393–402 (1991).
- Morucci, J. P., Granié, M., Lei, M., Chabert, M. & Marsili, P. M. *Physiol. Meas.* **A16**, A123–A128 (1995).
- Goble, J., Chenney, M. & Isaacson, D. *Appl. Comput. Electromagn. Soc. J.* **7**, 128–147 (1992).
- Brown, B. H. et al. (spec. iss. 1) *Innov. Tech. Biol. Med.* **15**, 1–8 (1994).
- Brown, B. H. & Seagar, A. D. *Clin. Phys. Physiol. Meas.* **A8**, 91–97 (1987).
- Geselowitz, D. B. *IEEE Trans. biomed. Engng* **18**, 38–41 (1971).
- Kotre, C. J. *Clin. Phys. Physiol. Meas.* **10**, 275–281 (1989).
- Barber, D. C. *Clin. Phys. Physiol. Meas.* **10**, 368–370 (1989).
- Witsoe, D. A. & Kinnen, E. *Med. Biol. Engng.* **5**, 239–248 (1967).
- Albert, A. *Regression and the Moore-Penrose Pseudo-inverse* (Academic, New York, 1972).
- Golub, G. H. & Reinsch, C. *Numer. Math.* **14**, 403–420 (1970).
- Hansen, P. C. *Numer. Alg.* **6**, 1–35 (1994).

ACKNOWLEDGEMENTS. This work was supported by Action Research and EPSRC.

Unrealistic desiccation of marine stratocumulus clouds by enhanced solar absorption

Andrew S. Ackerman & Owen B. Toon

NASA Ames Research Center, National Aeronautical and Space Administration, Moffett Field, California 94035, USA

The absorption of solar radiation by clouds affects the distribution of heat that drives atmospheric and ocean circulations. Many investigators have measured cloud solar absorption values exceeding theoretical estimates, although the discrepancies have been regarded as inconclusive¹. But more recent measurements indicate that clouds absorb up to three times as much solar energy than conventional theory predicts^{2,3}. Marine stratocumulus clouds are particularly sensitive to solar absorption because marine boundary-layer mixing is typically driven by cloud radiative processes. Here we present model simulations of a stratocumulus-topped marine boundary layer, incorporating different levels of solar absorption. With conventional absorption, the simulations reproduce observed cloud behaviour. But those with artificially enhanced absorption result in an unrealistic daytime depletion of cloud water, because a reduction in cloud radiative cooling results in decreased boundary-layer mixing. Moreover, we show that it is unlikely that liquid water or any plausible dissolved material can absorb the energy required by the recent measurements of solar absorption. Our results therefore indicate that either enhanced solar absorption occurs only in clouds other than marine stratocumulus, or the enhancement of cloud solar absorption indicated by recent measurements^{2,3} is overestimated.

Many models of the stratocumulus-topped marine boundary layer show that vertical mixing is driven by radiative cooling near the cloud top^{4–10}. These models also show that absorption of solar radiation by clouds can lead to reduced boundary-layer mixing, which restricts the supply of water vapour and results in a daytime thinning of cloud layers. Here we assess the effects of enhanced solar absorption on numerical model simulations of the stratocumulus-topped marine boundary layer, and we address the issue of whether any realistic mechanism can account for the measured enhancement of cloud solar absorption.

To investigate the effects of enhanced solar absorption by clouds, we use a one-dimensional model that treats aerosol and cloud microphysics, radiative transfer and turbulent mixing in the stratocumulus-topped marine boundary layer¹⁰. With conventional treatment of solar absorption, model-predicted profiles of thermodynamics, cloud microphysics, radiative fluxes and turbulent fluxes compare favourably with airborne measurements of a marine stratocumulus cloud deck^{10,11}. Profiles of liquid water are shown in Fig. 1a. Vertical mixing was driven primarily by the turbulent buoyancy flux due to radiative cooling near the cloud top

(Fig. 1b). The broadband solar absorption for the boundary layer was measured as $\sim 80 \text{ W m}^{-2}$, which exceeded the 53 W m^{-2} predicted by the model; the discrepancy falls within the measurement uncertainties and is primarily due to small differences between the modelled and measured cloud microstructures.

We have explored model sensitivities and found that the model behaves similarly to other one-dimensional models and responds realistically to changes in boundary conditions¹⁰. Our model results also agree with horizontal averages taken from a three-dimensional simulation of the same stratocumulus deck¹². Although our model generally reproduces measurements of marine stratocumulus, simplifications are made in a one-dimensional approach. For example, our radiative transfer scheme does not treat broken clouds.

For a baseline case (with conventional solar absorption), we use our model simulation of the marine stratocumulus measured by Nicholls^{10,11}. To quantify cloud solar absorption, recent studies have used R , the ratio of cloud shortwave (broadband) radiative forcing at the surface to that at the top of the atmosphere (cloud radiative forcing is defined as the difference between the cloudy and clear-sky net fluxes). To calculate clear sky fluxes we removed all droplets from the model domain at 12:00 (local noon), yielding $R = 1.16$ for the baseline case. This cloud forcing ratio corresponds to an increase in broadband solar absorption for the entire atmospheric column from 216 W m^{-2} in the clear sky to 282 W m^{-2} in the cloudy sky, or 19% to 25%, respectively, of the solar insolation at the top of the atmosphere.

Although this cloud forcing ratio is in the upper range of theoretically expected values¹³, some recent measurements indicate that R is much higher, presumably owing to enhanced solar absorption by clouds. Using a global network of surface radiometers and satellite measurements, Cess *et al.*² reported an average of $R = 1.5$. But applying the same analysis to a different data set, Li *et al.*¹⁴ found such a large average value in the tropics only. Pilewskie and Valero³ derived a large value of R (comparable to that reported by Cess *et al.*²) using identical radiometers simultaneously flown above and below cloud layers high in the tropical troposphere. In contrast, Hayasaka *et al.*¹⁵ collected

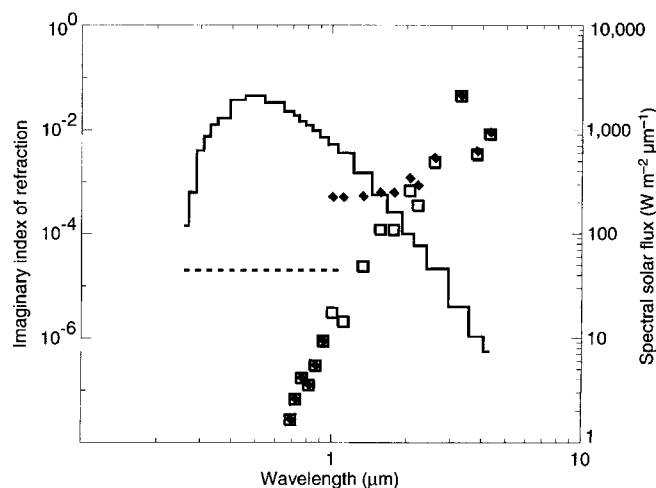


FIG. 2 Imaginary part of the index of refraction for liquid water (left ordinate). Squares, standard values¹⁷⁻¹⁹ used in the model; diamonds, values used in the enhanced simulations; the dashed line is at a value of 2×10^{-5} (equivalent to the minimum value required for a broadband enhancement). For reference the spectral solar flux is overlaid as a solid line (right ordinate).

similar measurements above and below stratocumulus layers, corrected the measurements for cloud heterogeneity¹⁶, and found no excess cloud absorption.

To enhance solar absorption in our model, we increase the imaginary part of the refractive index of liquid water (k) for wavelengths between 1 and $4.5 \mu\text{m}$. We use Mie theory to revise the droplet absorption efficiencies, then recalculate the radiative fluxes for the baseline simulation at noon. To attain $R = 1.5$ with a uniform increase in k between 1 and $4.5 \mu\text{m}$ requires an addition of 5×10^{-4} to the standard values¹⁷⁻¹⁹ as depicted in Fig. 2. In this case, the cloudy-sky column absorbs 36% of the solar insolation.

Next we run a model simulation starting from a cloudless initial state, but with the revised droplet absorption efficiencies (we will refer to this simply as the 'enhanced simulation'). A comparison of the simulations is shown in Fig. 3. In the baseline simulation, slight minima of cloud water and optical depth occur around noon because solar absorption partially offsets longwave cooling near the cloud top. But in the enhanced simulation, much more pronounced daytime minima develop and persist from about 10:00 to about 18:00 (also evident in the profiles of cloud water in Fig. 1a). The cloud layer in the enhanced simulation is depleted of moisture owing to a significant reduction in vertical mixing (Fig. 1b), resulting from the enhanced solar absorption. Although the revised droplet optical properties result in $R = 1.5$ for the baseline cloud, in the enhanced simulation the forcing never reaches that value (at noon it is only 1.33) owing to a negative feedback between increased absorption and decreased cloud thickness.

To see if the drastic daytime depletion of cloud water can be avoided, we have evaluated the effects of uncertainties in model inputs. The condensation coefficient describes the fraction of water molecules that stick after impinging on a droplet. Running the enhanced simulation again with a condensation coefficient of 0.035 (rather than 1.0), the cloud layer again thinned drastically. Other input uncertainties include the subsidence rate and the sea surface temperature. We ran three further variations of the enhanced simulation: (1) no subsidence, (2) sea surface temperature increased by 1 K, and (3) no subsidence and the sea surface temperature increased by 1 K. In all three simulations, the cloud layer again thinned drastically. These results verify that the cloud-thinning effects of enhanced solar absorption are robust with respect to model uncertainties.

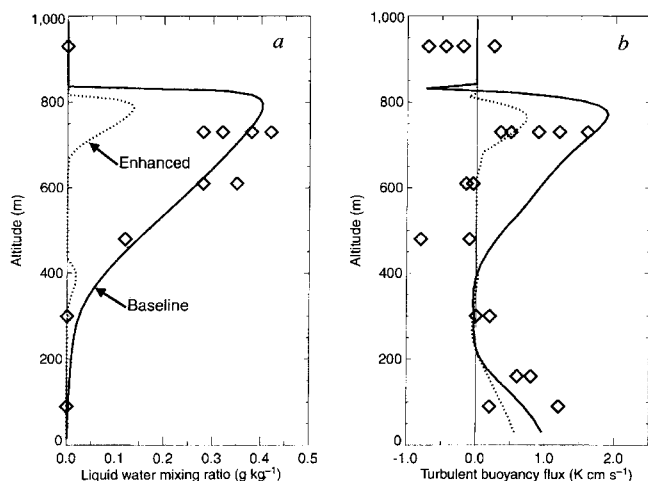


FIG. 1 Comparison between measurements and modelled profiles of liquid water mixing ratio (a), and turbulent buoyancy flux (b). The diamonds are measurements taken in a stratocumulus deck around noon over the North Sea during July 1982 (ref. 11); the solid (dotted) lines are the model output at 12:00 for the simulation with conventional (enhanced) solar absorption. The model simulations were initialized with cloudless conditions at midnight and run for 24 hours with the observed boundary conditions (during which a persistent cloud layer formed). In additional comparisons, the measured droplet distributions and drizzle fluxes compared favourably with the measurements¹⁰.

We have shown that enhanced solar absorption leads to an unrealistic daytime depletion of cloud water; next we explore possible mechanisms for such absorption. The imaginary refractive index of droplets with dissolved absorbing material varies linearly with the molar concentration of the dissolved absorber (this linear variation is commonly used in laboratory studies; its basis is found in the dispersion equations for the dielectric constant²⁰). Assuming that the dissolved material is extremely absorbing, so that $k = 1$ at a concentration of 1 M, the molar concentration of a dissolved absorber required to obtain the observed cloud forcing ratio is 5×10^{-4} . However, ionic concentrations in cloud water are generally $<10^{-4}$ M (refs 21 and 22). Exceptions are Cl^- , Na^+ , SO_4^{2-} and Mg^{2+} in some marine rain-water samples, and numerous inorganic ions in fogs. So even if we make a very generous assumption about the absorption by dissolved species, they are rarely found in concentrations high enough to provide the required absorption.

Another possibility is that the conventional k values for liquid water are systematically low. The measurement uncertainties in k are $\sim 10\%$ (refs 17–19), yet increasing k by a factor of 10 results in $R = 1.32$ for the baseline cloud. Hence uncertainties in k cannot account for the recently reported enhancement of solar absorption ($R = 1.5$). But to explore the effects of an intermediate

enhancement we ran a simulation with k increased by a factor of 10. The drastic daytime thinning of the cloud layer occurred again but was slightly delayed.

Next we consider a broadband enhancement due to suspended material such as black carbon (properties described in ref. 23). We assume that the carbon is symmetrically distributed as a shell around each water droplet, which maximizes its absorption efficiency (thereby requiring the minimum amount of carbon), and for simplicity we assume a constant volume fraction of carbon in each droplet. Revised scattering and absorption efficiencies are then calculated²⁴. To attain $R = 1.5$ in the baseline cloud requires a volume fraction of 4×10^{-5} , which exceeds by orders of magnitude the amounts of black carbon measured in clouds influenced by urban pollution²⁵ and in rain water affected by biomass burning²⁶. Together with a modelled liquid water path of 133 g m^{-2} , the volume fraction requires a black carbon concentration of $6 \mu\text{g m}^{-3}$ in the boundary layer (all of it in cloud droplets). Such high concentrations are sometimes exceeded in heavily polluted regions, but rarely so²⁷. (For comparison we assumed a fixed volume of carbon in each droplet and repeated the calculation; the same total concentration of carbon was required).

A broadband absorber can also be ruled out by commonplace observations. Prescribing a minimum value of $k = 2 \times 10^{-5}$ for liquid water also yields $R = 1.5$ for the baseline cloud. The transmission of light of wavelength λ through a uniform absorbing layer of thickness h is given by $\exp(-4\pi k h / \lambda)$. Hence a puddle of water 1 cm deep with such absorption would transmit only 1.5% of the incident light at a visible wavelength of $0.6 \mu\text{m}$. Clearly, rain water is much more transparent.

We find that there are no plausible mechanisms for cloud water to provide the absorption recently suggested by measurements. The uncertainties in the measured optical constants are insufficient; black carbon is not found in high enough concentrations (except in severely polluted air), and any broadband absorber would violate the commonplace observation that centimetre-thick layers of water are transparent. Also, there are no known dissolved species in water with high enough concentrations to account for the absorption (with the exception of special cases such as fog), even assuming the dissolved species are extremely good absorbers.

Our model results may be related to the shortcomings of our one-dimensional model¹⁰, although we are unaware of any systematic reasons for this to be the case. We recommend that our results be verified with three-dimensional models, but we anticipate that other models of radiatively driven cloud layers will produce similarly unrealistic results with enhanced solar absorption. We therefore conclude that either radiatively driven cloud models are less realistic than previously thought, or that the enhancement of cloud solar absorption indicated by recent measurements has been overestimated or does not apply to marine stratocumulus. □

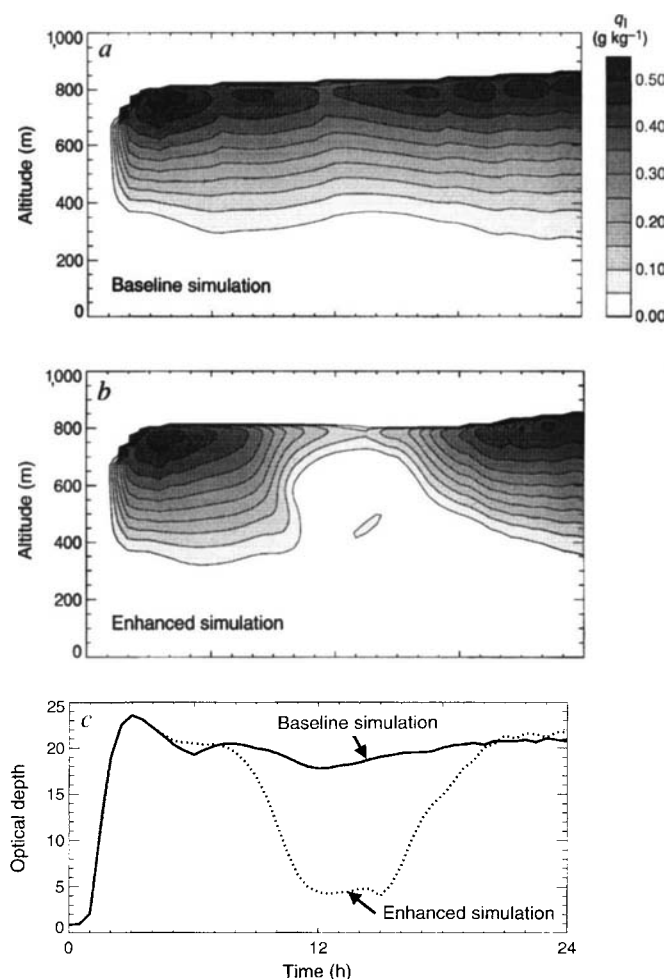


FIG. 3 Evolution of water mixing ratio (q_l , in units of g kg^{-1}) with time for the baseline simulation (a) and the enhanced simulation (near-infrared case; b). c, Evolution of optical depth (at $0.6 \mu\text{m}$) of the modelled boundary layers. Line patterns are the same as in Fig. 1. The maxima of liquid water and optical depth at 03:00 in both simulations are due to a burst of boundary-layer mixing caused by cloud formation. A transient lower cloud develops in the enhanced simulation owing to accumulation of vapour in the surface mixed layer.

Received 13 September 1995; accepted 9 February 1996.

- Stephens, G. L. & Tsay, S.-C. *Q. J. R. met. Soc.* **116**, 671–704 (1990).
- Cess, R. D. et al. *Science* **267**, 496–499 (1995).
- Pilewskie, P. & Valero, F. P. *J. Science* **267**, 1626–1629 (1995).
- Lilly, D. K. *Q. J. R. met. Soc.* **94**, 292–309 (1968).
- Bougeault, P. *J. atmos. Sci.* **42**, 2826–2843 (1985).
- Chen, C. & Cotton, W. R. *J. atmos. Sci.* **44**, 2951–2977 (1987).
- Turton, J. D. & Nicholls, S. Q. *J. R. met. Soc.* **113**, 969–1009 (1987).
- Duynkerke, P. G. *Mon. Weath. Rev.* **117**, 1710–1725 (1989).
- Rogers, D. P. & Koracin, D. J. *J. atmos. Sci.* **49**, 1473–1486 (1992).
- Ackerman, A. S., Toon, O. B. & Hobbs, P. V. *J. atmos. Sci.* **52**, 1204–1236 (1995).
- Nicholls, S. Q. *J. R. met. Soc.* **110**, 783–820 (1984).
- Kogan, Y. L., Khairoutdinov, M. P., Lilly, D. K., Kogan, Z. N. & Liu, Q. *J. atmos. Sci.* **52**, 2923–2940 (1995).
- Chou, M.-D., Arking, A., Otterman, J. & Ridgeway, W. L. *Geophys. Res. Lett.* **22**, 1885–1888 (1995).
- Li, Z., Barker, H. W. & Moreau, L. *Nature* **376**, 486–490 (1995).
- Hayasaka, T., Kikuchi, N. & Tanaka, M. *J. appl. Met.* **34**, 1047–1055 (1995).
- Ackerman, S. A. & Cox, S. K. *J. appl. Met.* **20**, 1510–1515 (1981).
- Painter, L. R., Birkhoff, R. D. & Arakawa, E. T. *J. chem. Phys.* **51**, 243–251 (1969).
- Palmer, K. F. & Williams, D. J. *opt. Soc. Am.* **64**, 1107–1110 (1974).
- Downing, H. D. & Williams, D. J. *Geophys. Res.* **80**, 1656–1661 (1975).
- Bohren, C. F. & Huffman, D. R. *Absorption and Scattering of Light by Small Particles* Ch. 9 (Wiley, New York, 1983).

21. Galloway, J. N., Likens, G. E., Keene, W. C. & Miller, J. M. *J. geophys. Res.* **87**, 8771–8786 (1982).
22. Warneck, P. *Chemistry of the Natural Atmosphere* 405–406 (Academic, London, 1988).
23. Chylek, P., Ramaswamy, V. & Cheng, R. *J. J. atmos. Sci.* **41**, 3076–3084 (1984).
24. Toon, O. B. & Ackerman, T. P. *Appl. Opt.* **20**, 3657–3660 (1981).
25. Twohy, C. H., Clarke, A. D., Warren, S. G., Radke, L. F. & Charlson, R. J. *J. geophys. Res.* **94**, 8623–8631 (1989).
26. Cachier, H. & Ducret, J. *Nature* **352**, 228–230 (1991).
27. Cachier, H., Brémond, M.-P. & Baut-Ménard, P. *Nature* **352**, 371–373 (1989).

ACKNOWLEDGEMENTS. This work was supported by NASA and the US DOE. A.A. was supported by a Research Associateship from the US National Research Council.

Fluxes of CO₂ and water between terrestrial vegetation and the atmosphere estimated from isotope measurements

Dan Yakir & Xue-Feng Wang

Department of Environmental Sciences and Energy Research,
The Weizmann Institute of Science, Rehovot 76100, Israel

THE atmospheric budget of carbon compounds can be balanced only by invoking a significant 'missing sink' for carbon dioxide^{1–3}. Identifying this sink requires a knowledge of CO₂ fluxes at global and local scales. The former can be estimated from global averages of CO₂ concentration and isotope composition^{4,9}; local-scale measurements have been made by analysing individual eddies of air^{10,11}. In both cases, the net CO₂ exchange is the sum of two opposing fluxes: uptake by gross primary productivity and release by respiration. Here we show that these two components can be estimated separately at the local scale from small vertical gradients in ¹³C and ¹⁸O in atmospheric CO₂ above vegetation. By also analysing the ¹⁸O content of moisture in the air samples, we can estimate evapotranspiration rates, providing information on water exchange between the biosphere and atmosphere¹². We suggest that this approach can be extended to the regional scale.

Gradients in concentrations and isotopic compositions of CO₂ and water vapour above crop fields were measured with a 12-m mast that enabled measurement of wind speed and sampling of air at several heights above the canopy. Air was sucked either through a CO₂/H₂O infrared gas analyser to determine concentration gradients or through a cold trap and a 2-l flask to obtain samples of water vapour and CO₂ for determination of isotopic gradients. The results of measurements on a sunny midday over a wheat field are shown in Fig. 1. As expected, concentrations of CO₂ decreased, and concentrations of H₂O increased, towards the vegetation because of net photosynthetic CO₂ uptake and evapotranspiration, respectively. Similarly, ^δ¹³C values of the CO₂ increased towards the vegetation because of the discrimination against ¹³C associated with photosynthetic CO₂ fixation¹³, while ^δ¹⁸O values of the CO₂ increased because of isotopic exchange with ¹⁸O-enriched leaf water^{7,9,14}.

Variations in trace gas concentrations, such as shown in Fig. 1a,f, have been commonly used to estimate net fluxes between the land surface and the atmosphere^{15,16}. This aerodynamic approach relies on the assumption that momentum, heat and mass (of, for example, CO₂ and H₂O) are transported by eddies of turbulent air and therefore behave similarly in this context. Fluxes and gradients can therefore be related by the eddy diffusivity. Although net fluxes can be more directly estimated based on co-variance of vertical wind speed and concentrations in individual eddies of air^{11,17}, the need for fairly large samples of air for isotopic analysis made the aerodynamic approach more appropriate for the studies

reported here. The net fluxes of water vapour and CO₂ were calculated from the gradients according to:

$$J_x = -k \frac{\Delta C_x}{\Delta z} \quad (1)$$

where ΔC_x is the vertical difference in concentration C of a constituent x over the vertical distance Δz in the canopy boundary layer, and k represents the eddy diffusivity of the air calculated from wind speed profiles and surface characteristics. The results for net fluxes of CO₂ and water vapour are given in Table 1. Notably however, it is the gross fluxes that need to be considered for understanding the behaviour of the biological system and its response to environmental change.

The approach used here to partition the net CO₂ flux into its components is based on the assumption that an air sample taken at a height z above the canopy contains a mixture of air from the background atmosphere (indicated by subscript a) with air that has had CO₂ or H₂O added/removed by the surface biological system (b) according to:

$$C_z = C_a - C'_b \quad (2)$$

where a vertical gradient C_z can be converted to a flux (equation (1)). C'_b may (prime indicates a composite value) be further subdivided into respiratory and photosynthetic components of CO₂ and H₂O (discussed below). Owing to isotopic fractionations associated with biological exchange of CO₂ and H₂O, an isotopic mass balance can be approximated by:

$$C_z \delta_z = C_a \delta_a - C'_b \delta'_b \quad (3)$$

where isotopic abundance is represented in the familiar δ notation¹⁸ (which introduces a possible error in the order of 0.01‰ when calculating δ'_b).

The mixing analysis of equations (2) and (3) can be rearranged in a useful way¹⁹ as:

$$\delta_z = \delta'_b + M/C_z \quad (4)$$

where $M = (\delta_a - \delta'_b)C_a$ and the intercept δ'_b can be empirically obtained from a plot of δ_z versus $1/C_z$ (Fig. 1c, e). Applying this regression analysis to the atmospheric water vapour data (Fig. 1f–h) yielded an intercept (δ'_b) of -3.7‰ , representing the isotopic signature of the evapotranspiration flux. This value is similar to the mean ^δ¹⁸O value of stem water in the wheat field ($-3.1 \pm 0.3\text{‰}$, $n = 14$) as indeed is required by the widely made assumption of isotopic steady-state of leaf water (during which the ^δ¹⁸O values of input (stem) water, and output (transpired) water vapour are equal^{14,20}). Consistency with the regression analysis was also found for the CO₂ results.

The biological flux of CO₂, with an isotopic signature δ'_b , is composed of respiratory (r) and photosynthetic (p) components that can be independently estimated. Considering their specific isotopic signatures in the context of equation (3) leads to:

$$C_z \delta_z = C_a \delta_a - C_p \delta_p + C_r \delta_r \quad (5)$$

Equation (5) can be solved for C_p and (because $C'_b = C_p - C_r$) for C_r . Using these values, together with estimates of the eddy diffusivity, the gross photosynthetic and respiratory fluxes, J_p and J_r respectively, can now be calculated (see equation (1)) according to:

$$J_p = -k \frac{\Delta C_p}{\Delta z} = -k \frac{\Delta C'_b}{\Delta z} f_1 \quad (6)$$

and

$$J_r = -k \frac{\Delta C_r}{\Delta z} = -k \frac{\Delta C'_b}{\Delta z} f_2 \quad (7)$$

where $f_1 = (\delta'_b - \delta_r) / (\delta_p - \delta_r)$ and $f_2 = (\delta'_b - \delta_p) / (\delta_p - \delta_r)$. Notably, the δ values used in the equations above represent either ^δ¹⁸O or ^δ¹³C, each dominated by different processes and yielding independent estimates of J_p and J_r .

To apply the above approach at the field scale, we characterized the isotopic signatures of the CO₂ associated with photosynthesis,

For $\delta^{18}\text{O}$, the isotopic signature of the CO_2 associated with photosynthesis, δ_{p}^{18} , is influenced by catalysed oxygen exchange with water that occurs primarily in the chloroplast. Assuming that the $\delta^{18}\text{O}$ value of water in chloroplasts is similar to that of bulk leaf water (δ_{LW})¹⁴, we used δ_{LW} measured across the field ($9.9 \pm 0.8\text{‰}$, $n = 14$ for the wheat field reported in Fig. 1) to estimate the $\delta^{18}\text{O}$ value of CO_2 in equilibrium with chloroplast water²¹ before CO_2 assimilation in photosynthesis (δ_{ch}). This value was then used to estimate δ_{p}^{18} according to⁹:

where $\bar{a}_1$, the diffusional fractionation, was estimated to be 8.0‰ and $\varepsilon = C_{\text{ch}}/(C_{\text{a}} - C_{\text{ch}})$, calculated based on leaf scale gas exchange measurements of photosynthesis and stomatal conductance¹³ across the fields. Alternatively, the $\delta^{13}\text{C}$ value of leaf organics which is influenced by ε can also provide a time-integrated record of this parameter¹³. Notably, estimates of δ_{p}^{18} were consistent with δ_{b}^{18} values calculated from the regression analysis discussed above (Table 1).

$$\delta_r^{18} = (\delta_s + \varepsilon_{eq}) - \bar{a}_2 \quad (9)$$

where δ_s represents the $\delta^{18}\text{O}$ of soil and stem water (directly-measured), $\varepsilon_{\text{eq}} = (\alpha - 1)10^3$, where α is the temperature-sensitive $\text{CO}_2\text{-H}_2\text{O}$ equilibrium fractionation²¹, and $\bar{\alpha}_2$, the diffusional fractionation, was taken as the maximum value for molecular diffusion, 8.8‰, found useful for measurements in a grassland ecosystem²². The results are shown in Table 1. More experimental

Estimates of δ_r^{13} were based on the $\delta^{13}\text{C}$ value of plant organics sampled across the field (Table 1) which was, as expected in these mostly monoculture fields, very similar to that of total soil organics sampled at 40 cm depth at the centre of the fields. Significantly, these long-term mean $\delta^{13}\text{C}$ values were distinct from the short-term, midday δ_p^{13} values that were calculated based on leaf scale measurements carried out concurrently with the air sampling. As noted above for δ_p^{18} , estimates of δ_p^{13} were consistent with δ_b^{13} values obtained from the regression analyses (Table 1). Note that δ_p^{13} and consequently δ_b^{13} values were $\sim 10\text{‰}$ higher in the corn field than in wheat, as would be expected above C_4 plants. The much smaller differences in δ_p^{18} between the wheat and corn fields reflected small differences in leaf water enrichment and stomatal conductance (not shown).

Using the estimates of the isotopic signatures reported in Table 1, combined with the concentration measurements and concurrently estimated eddy diffusivity ($0.194 \text{ m}^2 \text{ s}^{-1}$ for the wheat field reported in Fig. 1), the fluxes J_p and J_r for each of the crop fields studied here could be calculated according to equations (6) and (7) (Table 1). Rates of net CO_2 uptake by the crop fields were consistent with commonly observed rates^{23,24}, and partitioning of the net CO_2 flux yielded rates of respiration comparable to those made by either extrapolation from night-time or soil-profile measurements^{22,25}. The isotopic approach showed that in wheat, a decrease in net CO_2 uptake at the end of the growing season (from 44.2 to $20.6 \mu\text{mol m}^{-2} \text{ s}^{-1}$) reflected a proportionally greater decrease in respiration (71%) than in photosynthesis (56%), possibly due to soil drying near the surface.

Interestingly, combining estimates of water vapour flux, $J_{N(H_2O)}$, with those for the gross photosynthetic flux, J_p (Table 1), can provide a gross-primary-productivity-related estimate of the

516 NATURE · VOL 380 · 11 APRIL 1996

TABLE 1 Estimated field-atmosphere fluxes

	J_N ($\text{mol m}^{-2} \text{s}^{-1}$)		^{18}O (‰)			^{13}C (‰)			J_p ($\mu\text{mol m}^{-2} \text{s}^{-1}$)		J_r ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	
	$\text{CO}_2 (\times 10^6)$	$\text{H}_2\text{O} (\times 10^3)$	δ'_p	δ_b	δ_r	δ'_b	δ_p	δ_r	^{18}O	^{13}C	^{18}O	^{13}C
Wheat 1	44.2	7.7	14.12	16.01	29.40	-29.36	-29.00	-27.40	50.4	54.1	-6.2	-9.9
Wheat 2	20.6	4.8	16.06	17.14	29.20	-28.47	-	-27.40	22.4	-	-1.8	-
Cotton	35.4	13.8	19.30	20.68	29.50	-25.91	-25.87	-25.40	40.9	37.8	-5.5	-2.4
Corn	27.6	7.5	17.59	18.88	30.40	-19.97	-15.67	-	30.7	-	-3.1	-

Estimated fluxes of CO_2 and water vapour exchanged between different crop fields and the atmosphere during midday. Net CO_2 and water vapour fluxes (J_N) were estimated from concentration gradients (see Fig. 1) and eddy diffusivity (estimated from wind speed profiles, measured with cup anemometers, and surface characteristics). Net CO_2 fluxes were partitioned to photosynthetic CO_2 uptake (J_p) and respiratory CO_2 release (J_r) by using analysis of the isotopic gradients in the canopy boundary layer to estimate δ'_b (Fig. 1), and estimates of the isotopic signatures of the CO_2 taken up in photosynthesis (δ_p) and released in respiration (δ_r). Estimates of δ_p and δ_r were based on analysis of water and organic matter from leaves, stems and soil samples collected across the fields concurrently with the air samples. Wheat 1 was measured on 20 February 1994 during peak activity; wheat 2 on 23 March at the end of the growing season.

vegetation water use efficiency ($J_p/J_{N(\text{H}_2\text{O})}$). Independent estimates of $J_{N(\text{H}_2\text{O})}$, say from energy balance¹⁵ or by incorporation of eddy correlation techniques¹⁷, may help in extracting J_p . $J_{N(\text{H}_2\text{O})}$ itself could also be evaluated by using isotopic values as surrogates for concentrations¹². In this case gradients and fluxes could be estimated based on $C_w = C_a(\delta_a - \delta_s)/(\delta_s - \delta_e)$, assuming $\delta'_b = \delta_s$ and the latter obtained from stem water (where C'_w substitutes for C_b when considering water). Consistency between estimates obtained in this way and those reported in Table 1 provided another check on the isotopic mass balance approach employed here (not shown).

The intermediate (field) scale chosen here allowed us to establish the quantitative relationships between isotopic variations in the atmosphere and specific biospheric processes, which are uncertain at the global scale and unrealistic in the laboratory. Expansion of such measurements to scales which are spatially and/or temporally larger may be challenging. Larger, heterogeneous systems will be increasingly difficult to characterize in terms of surface isotopic signatures, and will need appropriately scaled sampling strategies. Important prerequisites for the application of the isotopic approach are the existence of the differences between δ_r and δ_p and between δ_b and δ_a . Differences between δ_r and δ_p in ^{13}C may be small in established ecosystems where the $\delta^{13}\text{C}$ values of decomposing and newly fixed organic matter are very similar (although seasonal variations may help in this respect). In contrast, considerable differences in ^{18}O between δ_p and δ_r can be generally expected because of the large ^{18}O enrichment in leaf water²⁶. This may provide a significant advantage for ^{18}O over ^{13}C as tracer for partitioning leaf and soil CO_2 exchange (an advantage that may be restricted in some locations where geographical variations in the $\delta^{18}\text{O}$ values of environmental water²⁶ impose

similar δ_b^{18} and δ_a^{18} values). Research is clearly needed to further reduce uncertainties in estimating δ_r and δ_p . Specifically, isotopic fractionations associated with diffusion and CO_2 - H_2O equilibrium of soil CO_2 , as well as the isotopic signature of chloroplast water, are not well characterized at present. The specific ^{18}O signature of chloroplast water has, however, been addressed in two recent laboratory studies^{9,14}. In practice, it is often useful to rely on a hydrological model that predicts the $\delta^{18}\text{O}$ value of water undergoing evaporation based on ambient humidity and its $\delta^{18}\text{O}$ value^{27,28}. It was encouraging to find that transpiration from the crop fields canopies appeared to be at isotopic steady-state as required by the model, and that the evaporation model yielded δ_{LW} values that were generally similar to measured values when the observed gradients within the canopy boundary layer (for example, Fig. 1f, g) were considered. However, this approach does not fully account at present for variations in humidity and its $\delta^{18}\text{O}$ values within the canopy boundary layer, or for physiological effects such as those observed when different plant species growing at the same site have very different δ_{LW} values²⁶.

The usefulness of including isotopic measurements of CO_2 at the global scale has already been noted⁴⁻⁹, and complementing these studies with local-scale measurements will undoubtedly improve our understanding of the net CO_2 exchange between the biosphere and the atmosphere. But it may prove essential to evaluate the gross fluxes of photosynthesis and respiration in order to gain insights into ecosystem response to environmental changes (such as variations in temperature or atmospheric CO_2 concentrations²⁹). It is in this context that the isotopic approach, linked with existing regional-scale flux measurements relying on analysis of concentration alone, may have its greatest potential. □

Received 30 May 1995; accepted 1 March 1996.

1. Tans, P. P., Fung, I. Y. & Takahashi, T. *Science* **247**, 1431-1438 (1990).
2. Sundquist, E. T. *Science* **259**, 934-941 (1993).
3. Siegenthaler, U. & Sarmiento, J. L. *Nature* **365**, 119-125 (1993).
4. Tans, P. P., Berry, J. A. & Keeling, R. F. *Global biogeochem. Cycles* **7**, 353-368 (1993).
5. Ciais, P., Tans, P., Trolier, M., White, J. W. C. & Francey, R. J. *Science* **269**, 1098-1102 (1995).
6. Francey, R. J. *et al. Nature* **373**, 326-330 (1995).
7. Francey, R. J. & Tans, P. P. *Nature* **327**, 495-497 (1987).
8. Friedli, H., Siegenthaler, U., Rauber, D. & Oeschger, H. *Tellus* **39B**, 80-88 (1987).
9. Farquhar, G. D. *et al. Nature* **363**, 439-443 (1993).
10. Wofsy, S. C. *et al. Science* **260**, 1314-1317 (1993).
11. Grace, J. *et al. Science* **270**, 778-780 (1995).
12. Brunel, J. P., Simpson, H. J., Herczeg, A. L., Whitehead, R. & Walker, G. R. *Wat. Resour. Res.* **28**, 1407-1416 (1992).
13. Farquhar, G. D. & Lloyd, J. in *Stable Isotopes and Plant Carbon/Water Relations* (eds Ehleringer, J. R., Hall, A. E. & Farquhar, G. D.) 47-70 (Academic, New York, 1993).
14. Yakir, D., Berry, J. A., Giles, L. & Osmond, C. B. *Plant Cell Environ.* **17**, 73-80 (1994).
15. Thorn, A. S. in *Vegetation and the Atmosphere* (ed. Monteith, J. L.) 57-109 (Academic, London, 1975).
16. Monteith, J. L. & Unsworth, M. H. *Principles of Environmental Physics* 2nd edn (Arnold, London, 1990).
17. Unsworth, M. H. in *Plants and their Atmospheric Environment* (eds Grace, J., Ford, E. D. & Jarvis,

- P. G.) 11-138 (Blackwell, Oxford, 1981).
18. Hayes, J. M. *Spectra* **8**, 3-8 (1982).
19. Keeling, C. D. *Geochim. cosmochim. Acta* **24**, 277-298 (1961).
20. Wang, X. F. & Yakir, D. *Plant Cell Environ.* **18**, 1377-1385 (1995).
21. Friedman, I. & O'Neill, J. R. *Data of Geochemistry* 6th edn, ch. KK *Compilation of Stable Isotope Fractionation Factors of Geochemical Interest* (US Govt Printing Office, Washington DC, 1977).
22. Hesterberg, R. & Siegenthaler, U. *Tellus* **43B**, 197-205 (1991).
23. Denmead, O. D. in *Vegetation and the Atmosphere* (ed. Monteith, J. L.) 1-30 (Academic, New York, 1976).
24. Nobel, P. S. *Physiochemical and Environmental Plant Physiology* (Academic, San Diego, 1991).
25. Biscoe, P. V., Scott, R. K. & Monteith, J. L. *J. appl. Ecol.* **12**, 269-293 (1975).
26. Förstel, H. in *Proc. 3rd Int. Symp. Environmental Biogeochemistry and Geomicrobiology* (ed. Krumbein, W. E.) 811-824 (Ann Arbor Science, Michigan, 1978).
27. Craig, H. & Gordon, L. I. in *Proc. Conf. Stable Isotopes in Oceanographic Studies and Paleotemperatures* (ed. Tongiorgi, E.) 9-130 (Lab. of Geology and Nuclear Science, Univ. Pisa, 1965).
28. Flanagan, L. B., Comstock, J. P. & Ehleringer, J. R. *Pl. Physiol.* **96**, 588-596 (1991).
29. Keeling, C. D., Whorf, T. P., Wahlen, M. & van der Plicht, J. *Nature* **375**, 666-670 (1995).
30. Santrock, J. & Hayes, J. M. *Analyt. Chem.* **59**, 119-126 (1987).

ACKNOWLEDGEMENTS. We thank W. G. Zhao for help in estimating eddy diffusivities and E. Negriano for technical assistance. D.Y. holds the Roland Schaeffer Career Development Chair. This work was supported by the Israel Science Foundation and by a Joint German Israeli Research Programme.

Lower Cretaceous deposits trapped near the equatorial Mid-Atlantic Ridge

E. Bonatti^{*†}, M. Ligi^{*}, A. M. Borsetti^{*}, L. Gasperini^{*}, A. Negri[‡] & R. Sartori[‡]

^{*} Istituto Geologia Marina CNR, via P. Gobetti 101, 40129 Bologna, Italy

[†] Lamont Doherty Earth Observatory Columbia University, Palisades, New York 10964, USA

[‡] University of Bologna, Italy

ACCORDING to palaeogeographic reconstructions, the equatorial Atlantic Ocean basin started to open in the Cretaceous period, not earlier than 120 Myr ago^{1–3}. From this and a conventional description of the process of sea-floor spreading, one would expect the oldest oceanic crust in this region to be 120 Myr old, and to be found at the edges of the ocean basin. Contrary to these expectations, we report here the recovery of 140-Myr-old 'Maiolica'-type pelagic limestones from the centre of the basin, near the intersection of the Mid-Atlantic Ridge and the Romanche transform fault. The limestones occur within a tectonically deformed and uplifted sedimentary sequence, > 4 km thick and > 200 km long, which includes continent-derived quartzitic siltstones of Palaeocene and Eocene age. This sequence probably accumulated in a proto-Romanche transform valley that was connected to a palaeo-central-Atlantic basin, indicating that continental separation had already started here 140 Myr ago. The entrapment of these old deposits within younger ocean crust can be explained by repeated ridge jumping and transform migration during the evolution of the equatorial Atlantic.

The geology of the equatorial Atlantic is characterized by an east–west megashear belt where large transform faults offset the Mid-Atlantic Ridge (MAR). The largest is the Romanche fracture zone (FZ) that offsets the MAR by about 900 km for an estimated age offset of about 50 Myr (refs 4–6). It can be traced by satellite gravimetry to South American and African sheared continental margins and with approximately east–west offsets of the coastlines (Fig. 1). Hence, the Romanche has acted as a transform plate boundary since the early stages of continental separation.

A major transverse ridge runs subparallel to, and on the northern side of the Romanche FZ (Fig. 1). It reaches > 4 km above the predicted depth/age of the crust level in a stretch of several hundred kilometres located opposite the eastern ridge/transform intersection (RTI)⁵. Multichannel seismic reflection profiles and rock sampling demonstrated that the transverse ridge consists of uplifted slivers of oceanic lithosphere⁵. However, the seismic response of the transverse ridge changes drastically east of about 15° 30' W, roughly 100 km east of the RTI (Fig. 1), where it reveals a thick (> 4 s two-way travel time) sequence of stratified, tectonically deformed material (Figs 2 and 3). The recovery of consolidated limestones, quartzitic siltstones and biomicrites confirm that the seismic profiles imaged a sedimentary sequence, which we call the Romanche Sedimentary Sequence (RSS). Given that the acoustic P-wave velocity of this consolidated material is certainly > 2 km s⁻¹, the RSS is at least 4 km thick.

Two major seismic units can be identified within the RSS, separated by a reflector that probably marks an unconformity (Figs 2 and 3). The upper unit is layered, and folded and affected by roughly north–south overthrusts suggesting compression. Samples dredged where this unit is exposed on the sea floor are laminated (laminae are a few millimetres thick) tectonized biomicrites and quartz siltstones, made of coarse (~ 100–200 µm), well sorted angular quartz grains implying a close continental (granitic) source. They are probably contourites. The age of these

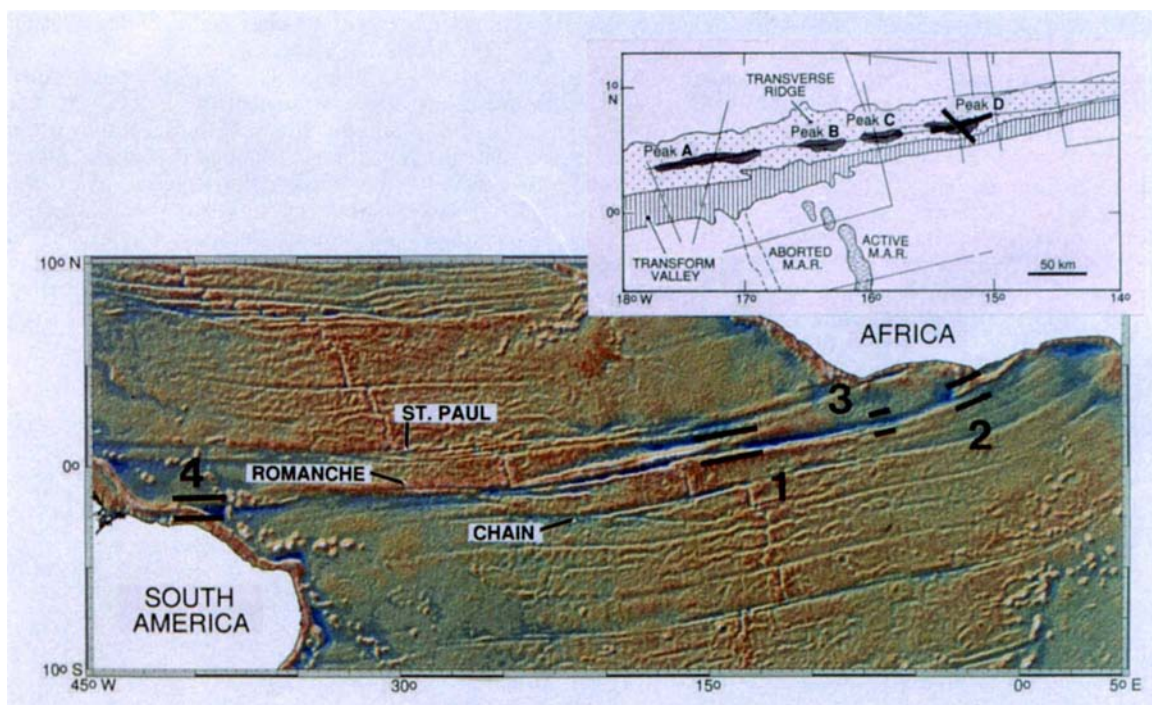


FIG. 1 Main figure, SeaSat, GeoSat and ERS-1 satellite gravity imagery of the equatorial Atlantic, version V7.2 (ref. 20). The axis of the Mid-Atlantic Ridge and the large fracture zones offsetting it can be identified. Four areas are identified along the Romanche FZ: 1, a portion of the Romanche FZ, where seismic reflection profiles show the transverse ridge is made of RSS; 2, Ivory Coast–Ghana sheared margin¹⁴; 3, outcrop of terrigenous siltites on

the transverse ridge¹⁹; 4, north Brazilian sheared margin¹⁵. Inset, schematic diagram of the Romanche eastern RTI area. Thin lines indicate tracks of multichannel seismic reflection profiles on the transverse ridge. Portions of profiles ROM 32 and ROM 43, shown in Figs 2 and 3, are represented by thicker lines. Peaks A, B, C and D are reliefs on the crest of the transverse ridge, described in ref. 5.

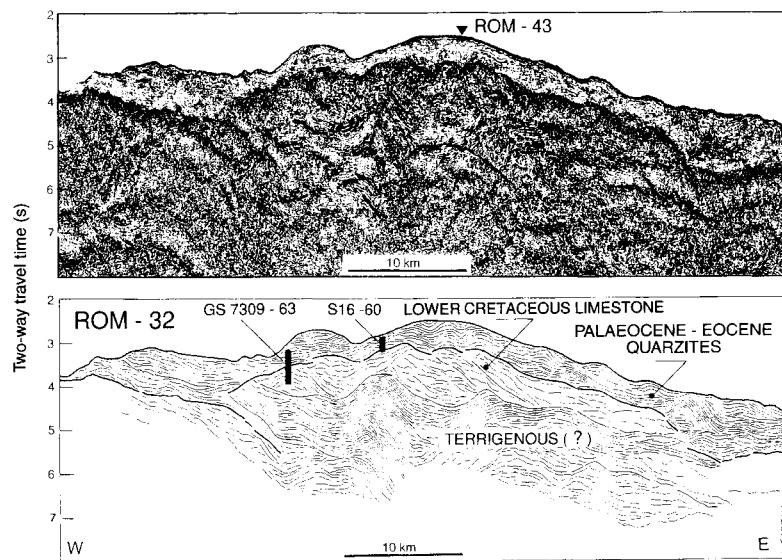


FIG. 2 Top panel, portion of an east–west seismic reflection profile (ROM 32) taken along the crest of the Romanche transverse ridge. Methods are similar to those described in ref. 5, except that three 210 in³ air guns were employed as sound source. Location of the profile is shown in Fig. 1 inset. The arrow points to the location where this profile is crossed by profile ROM 43, shown in Fig. 3. Different units are identified in the interpreted profile (bottom panel) on the basis of dredged samples. Dredging intervals are projected on the profile. Station GS7309-63 (0° 42' N; 15° 20' W; 2,270–2,950 m below sea level) released Maiolica limestones and Palaeocene/Eocene siltstones. Station S16-60 (0° 42.95' N; 15° 20.65' W; 2,250–2,450 m below sea level) released Palaeocene/Eocene siltstones. Limestones and siltstones were obtained also at Station S16-55, (0° 47.80' N, 14° 59.35' W; 3,300–3,500 m below sea level) located near the crest of the transverse ridge about 30 km east of site GS3709-63, outside the profile shown in this figure.

rocks, based on foraminiferal assemblages (with genera *Morozovella* and *Acarinina*, biostratigraphic zones P3 to P10–P14 of Blow⁷) and nannofossils (zones NP9 to NP17 of Martini⁸), is between Middle Palaeocene and Middle Eocene, that is, 65 to 55 Myr before present^{7–9}, although the lower strata of this unit may go back to the Late Cretaceous.

The lower unit shows deformed blocks, with overthrusts and with folds whose style and orientation is different from that of the upper unit. The upper part of the lower unit is not finely layered. Sampling of its roof released radiolarian-bearing dolomitized limestones containing a calpionellid microfossil population (with *Calpionella alpina*, *Calpionellopsis oblonga* and *Calpionellopsis simplex* and absence of *Crassicalearia* and *Calpionellites*) of Berriasian (Early Cretaceous) age, that is, ~140 Myr before present¹⁰. These samples resemble the Lower Cretaceous Maiolica formation, distributed in peri-Tethys regions^{11–13}, and found also at both edges of the central Atlantic close to the base of the continental slope, as in the Cape Verde and Blake–Bahamas basins^{12,13}. However, they are strongly recrystallized, with most of the original micritic calcite having been transformed into dolomite. This has probably prevented the preservation of the nannofossils that are frequently found in Maiolica-type limestones, and also makes it impossible to extract radiolarian tests (present in these samples, as is usual for the Maiolica) for an independent age determination. The limestones grade down into stratified, deformed, probably detrital deposits of unknown age (Figs 2 and 3). The base of the RSS has not been detected in our profiles.

The occurrence of the RSS near the Romanche eastern RTI is puzzling. If the RSS were underlain by oceanic crust, the predicted age of this crust (assuming a spreading rate of 1.7 cm yr^{–1} (ref. 2) and the present ridge/transform geometry) should be around ~55 Myr. However, the age of the RSS goes back to at least 140 Myr, when the equatorial Atlantic region is thought to have been still in a pre-oceanic rift phase^{1–3,14}.

The opening of the equatorial Atlantic involved, over a period of several million years, continent/continent and then continent/ocean transform motion^{1,3,14} resulting in sheared continental margins in west Africa and Brazil. The Romanche FZ merges with transpressive structural highs on the Brazilian¹⁵ and African¹⁴ sheared margins, both made of continent-derived deformed Cretaceous deposits^{14,16}. Transensional pull-apart basins also formed along both margins^{14,15}.

A possible model for the origin of the RSS implies deposition into a 'proto-Romanche' transform valley in its continent/continent, continent/ocean and ocean/ocean stages from the Early

Cretaceous to the Eocene. Continent-derived detritus accumulated in the deepest areas of the valley (transtensional pull-apart basins). Lower Cretaceous pelagic limestones formed on topographic highs within the continent/continent transform depression (Fig. 4).

The presence of 140-Myr-old Maiolica-type pelagic limestones as far south as the Romanche FZ has important implications for the opening of the equatorial Atlantic, hitherto thought to have started not earlier than the Aptian stage, ~120 Myr before present^{1–3,14}. It requires that the Romanche continent/continent transform depression was filled already in the Early Cretaceous by

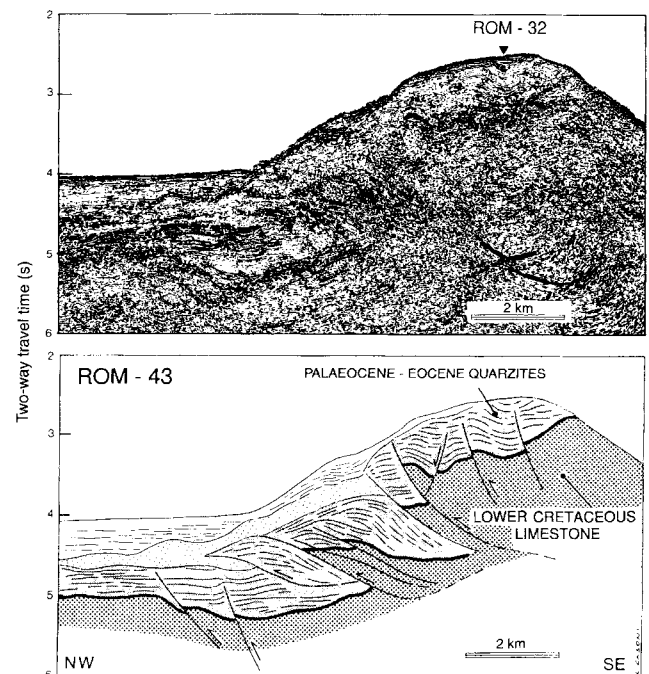


FIG. 3 Top panel, northwest–southeast seismic reflection profile (ROM 43) across the Romanche transverse ridge. Location of the profile is shown in Fig. 1 inset. The arrow points to the location where this profile is crossed by profile ROM 32, shown in Fig. 2. Bottom panel, the interpreted profile.

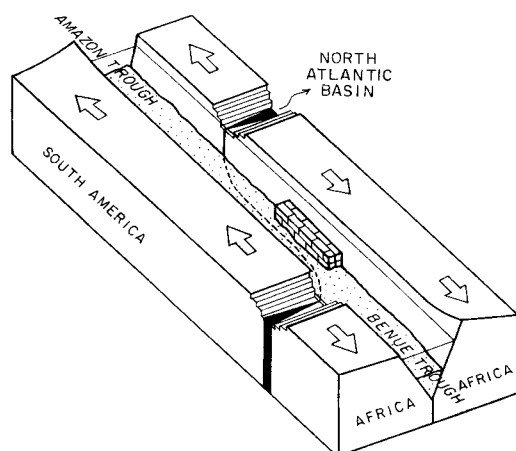


FIG. 4 Idealized representation of the equatorial Atlantic region in the Early Cretaceous. A palaeo-Romanche continent/continent transform is active along an equatorial fracture zone depression which is filled with oxygenated, circulating sea water and receives detrital sediments from the adjacent continents. The depression communicates to the northwest with a palaeo-North Atlantic basin. Limestones can accumulate in topographic highs within the depression.

oxygenated, circulating, relatively deep sea water, that communicated to the northwest with the central/north palaeo-Atlantic basin (Fig. 4). The RSS Palaeocene/Eocene unit may represent contourites fed by continent-derived turbidites channelled into a Romanche FZ valley that in the early Cenozoic was probably a major avenue of communication between north and south Atlantic waters.

The hypothesis that the RSS accumulated in an early Romanche FZ valley is consistent with its present areal distribution that in a north-south direction is limited essentially to the width of the transverse ridge (~50 km) while in east-west profiles the RSS was identified along the transverse ridge at least as far east as 13° 30' W, for a distance of > 200 km (area 1 in Fig. 1). Allochthonous continent-derived deformed quartzitic siltstones of unknown age, similar to those recovered from the RSS to the west and from the Ivory Coast/Ghana marginal ridge to the east, were recovered at 06° 30' W (area 2 in Fig. 1), where the summit of the transverse ridge emerges from the pelagic sediment blanket¹⁶. These observations suggest that lenses of continent-derived deposits are emplaced in a narrow strip along the Romanche FZ from near the eastern RTI to the Ivory Coast/Ghana sheared margin, a distance of more than 1,500 km (Fig. 1).

The RSS was uplifted in post-mid-Eocene time to form the transverse ridge, probably due to thrust faulting along the transform boundary, as suggested by seismic reflection profiles (Fig. 3). Transpressional and transtensional events related to changes in the geometry of equatorial Atlantic plate boundaries may have been the cause⁵. Two interpretations are possible. The layered deposits below the Lower Cretaceous limestone could be the same Palaeocene/Eocene terrigenous unit found at the top of the sequence, repeated by doubling of the sequence due to thrusting (Fig. 3). Alternatively, the deposits below the limestones represent older, possibly Upper Jurassic/Lower Cretaceous continental deltaic/lacustrine sediments, equivalent to those drilled in the Ivory Coast/Ghana margin by ODP leg 159¹⁷. The different style and probably longer history of deformation shown by the lower unit favours the latter hypothesis.

According to plate-tectonic theory, the age of the oceanic lithosphere increases with distance from the zero-age axis of a mid-ocean ridge. How then could ancient sequences deposited in the earliest stages of opening become trapped as allochthonous rafts in younger oceanic crust close to the RTI? The answer may lie in the peculiar geology of the equatorial Atlantic, characterized

by a very unstable ridge/transform geometry, with repeated ridge jumping and transform migration, particularly well documented at the Romanche FZ⁵. Both ridge jumping and transform migration provide mechanisms by which blocks of lithosphere may be transferred from the African to the South American plate or *vice versa*, reversing their sense of motion in what has been called oscillatory spreading¹⁸. Fragments of older oceanic lithosphere, or even of continental margin, could then be trapped within younger lithosphere. St Peter Paul Island might expose another such allochthonous fragment¹⁹.

Our results suggest that the simple plate-tectonic scheme of rifting and drifting inferred to explain the opening of the Atlantic requires added complexities in the equatorial region, in order to account for changes in ridge/transform geometry, intense vertical tectonics, and entrapment of old continental-margin deposits within younger lithosphere close to the axis of accretion. □

Received 10 October 1995; accepted 27 February 1996.

1. Rabinowitz, P. D. & LaBrecque, J. F. *J. geophys. Res.* **84**, 5973–6002 (1979).
2. Cande, S. C., LaBrecque, J. L. & Haxby, W. F. *J. geophys. Res.* **93**, 13479–13492 (1988).
3. Nurburg, D. & Muller, R. D. *Tectonophysics* **191**, 27–55 (1991).
4. Heezen, B. C., Burce, E. T., Hersey, J. B. & Tharp, M. *Deep-Sea Res.* **11**, 11–33 (1964).
5. Bonatti, E. et al. *J. geophys. Res.* **99**, 21779–21802 (1994).
6. Searles, R. C., Thomas, M. V. & Jones, E. J. W. *Mar. geophys. Res.* **16**, 427–453 (1994).
7. Blow, W. H. *The Cenozoic Globigerinida* Vol. 3, 1413 (Brill, Leiden, 1979).
8. Martini, E. in *Proc. 2nd Int. Conf. Planktonic Microfossils* Vol. 2, 739–785 (Technoscience, Rome, 1971).
9. Berggren, W. A. *Thaia* **5**, 195–215 (1972).
10. Remane, J. in *Plankton Stratigraphy* (eds Bolli, H. M., Saunders, J. B. & Perch-Nielsen, K.) 555–572 (Cambridge Univ. Press, 1985).
11. Bernoulli, D. & Jenkyns, H. C. in *Modern and Ancient Geosynclinal Sedimentation* (eds Dott, R. H. & Shaver, R. H.) 129–160 (Special Publ. 19, Soc. of Economic Paleontologists & Mineralogists, 1974).
12. Bernoulli, D. *Init. Rep. DSDP Leg 11*, 801–871 (1974).
13. Robertson, A. H. F. & Blüfnick, D. M. *Init. Rep. DSDP Leg 76*, 795–828 (1984).
14. Mascle, J. & Blarez, E. *Nature* **326**, 378–381 (1987).
15. Zalan, P. V., Nelson, E. P., Warme, J. E. & Davis, T. L. in *Strike Slip Deformation, Basin Formation and Sedimentation* (eds Biddle, K. T. & Christie-Blick, N.) 143–158 (Spec. Publ. 37, Soc. of Economic Paleontologists & Mineralogists, 1985).
16. Honnorez, J., Villeneuve, M. & Mascle, J. *Mar. Geol.* **117**, 237–251 (1994).
17. ODP Leg 159 Scientific Party *Eos* **76**, 299 (1995).
18. Bonatti, E. & Crane, K. *Nature* **300**, 343–345 (1982).
19. Bonatti, E. *Nature* **345**, 800–802 (1990).
20. Sandwell, D. T. & Smith, W. H. *Eos* **73**, 133 (1992).

ACKNOWLEDGEMENTS. We thank G. Bortoluzzi who had a major role in organizing the field work, and K. Rohr for constructive comments on the manuscript. This research is part of PRIMAR (Russian-Italian Mid Atlantic Ridge Project) and was supported by the Italian Consiglio Nazionale delle Ricerche.

Control of root growth and development by cyclin expression

Peter Doerner, Jan-Elo Jørgensen*, Ratha You, Johannes Steppuhn* & Chris Lamb

Plant Biology Laboratory, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, USA

Root development is plastic, with post-embryonic organogenesis being mediated by meristems¹. Although cell division is intrinsic to meristem initiation, maintenance and proliferative growth, the role of the cell cycle in regulating growth and development is unclear. To address this question, we examined the expression of *cdc2* and *cyc* genes, which encode the catalytic and regulatory subunits, respectively, of cyclin-dependent protein kinases that control progression through the cell cycle². Unlike *cdc2*, which is expressed not only in apical meristems but also before lateral root initiation³ in quiescent, pericycle cells arrested in the G2

* Present addresses: Gene Expression Laboratory, Department of Chemistry, Molecular Biology and Plant Physiology, University of Aarhus, Gustav Wiedsvej 10, 8000 Aarhus, Denmark (J.-E.J.); Institut für Physiologische Chemie, Uni-Klinikum Essen, Hufelandstr. 55, 45147 Essen, Germany (J.S.).

phase of the cell cycle⁴, *cyc1At* transcripts accumulate specifically in dividing cells immediately before cytokinesis. Ectopic expression of *cyc1At* under the control of the *cdc2aAt* promoter in *Arabidopsis* plants markedly accelerates growth without altering the pattern of lateral root development or inducing neoplasia. Thus cyclin expression is a limiting factor for growth, which in turn drives indeterminate development of the root system.

The levels of *cdc2* messenger RNA and p34^{cdc2} protein did not change markedly after stimulation of lateral-root initiation by the auxin indoleacetic acid (IAA) (Fig. 1a). Hence, whereas *cdc2* expression is correlated with the competence to divide, root growth and initiation of lateral roots do not appear to be limited by the abundance of the p34 catalytic subunit of cyclin-dependent protein kinase; moreover, ectopic expression of *cdc2* in transgenic *Arabidopsis* fails to perturb growth or development⁵.

In contrast, IAA treatment of *Arabidopsis* roots induced expression of several *cyc* genes over the low basal levels; in particular, *cyc1At* mRNA, which encodes a mitotic cyclin⁶, rapidly increased 15–20-fold (Fig. 1b). *In situ* hybridization showed that, unlike *cdc2*, *cyc1At* transcripts were not detectable in quiescent pericycle cells, but accumulated in single, cytoplasmically dense cells of incipient lateral root primordia. In the emergent organ, *cyc1At* was expressed exclusively in the meristem (Fig. 2a–d). Moreover, crucifer roots consist of long cell files that arise by transverse divisions followed by longitudinal expansion⁷, and within such a continuous spatial display of sequential cell-division phases,

cyc1At transcripts accumulated only in large cells immediately before cytokinesis, declining to background levels in adjacent small daughter cells (Fig. 2e, f). A similar, stringent spatio-temporal relationship of cyclin expression and mitosis was seen in *Antirrhinum* shoot apical meristems⁸.

The close correlation between *cyc1At* expression and cell division during growth of the root apical meristem and the

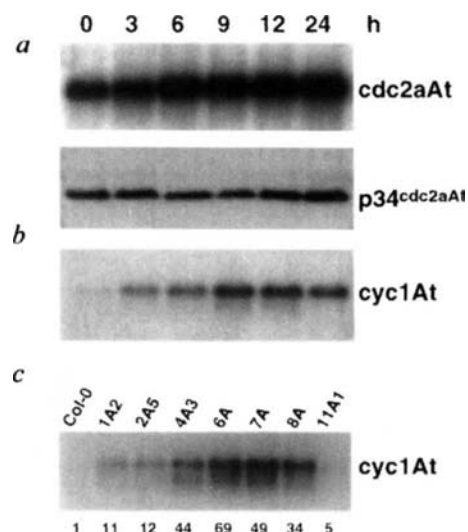


FIG. 1 Steady-state levels of a, *cdc2aAt* mRNA and p34 protein; b, *cyc1At* mRNA during IAA induction of lateral root meristems; c, *cyc1At* mRNA in selected non-induced transgenic lines; normalized transcript levels relative to wild-type are indicated. Col-0, wild type; 1A2, 2A5, 4A3, 11A1: T2 homozygous; 6A, 7A, 8A: T1 heterozygous transgenic lines. *cyc1At* mRNA levels in the lines 4A3, 6A, 7A, 8A, and 3A (not shown) exceed those of IAA-induced wild-type roots.

METHODS. *Arabidopsis* seedlings (ecotype Columbia) were grown in 20 ml MS medium²⁴. Plants, 8–10 days old, were transferred to MS medium buffered with 50 mM potassium phosphate, pH 5.5, and initiation of lateral roots was stimulated by addition of IAA to 10 μ M (non-dissociated IAA). Roots were collected at the time indicated and total RNA and protein isolated. 500 ng poly(A)⁺ RNA was separated on 1% formaldehyde gels²⁵, transferred to Nytran membranes (Schleicher and Schüll) and hybridized to ³²P-labelled probes corresponding to nucleotides (nt) 674–1,004 of *cyc1At*⁶, or nt 661–1,386 of *Arabidopsis cdc2aAt*²⁶, followed by hybridization with nt 2,576–2,824 of *Arabidopsis UBQ3*²⁷ for normalization. Blots were quantified with a Molecular Dynamics Phosphorimager. *cyc1At* is a single-copy gene in *Arabidopsis* (data not shown). Total protein was separated on 12% SDS-PAGE and transferred to PVDF membranes. p34^{cdc2aAt} was detected with serum raised in rabbits against the peptide YFKDLGGMP, corresponding to amino acids 286–294, and visualized by enhanced chemiluminescence (Amersham).

TABLE 1 Comparison of root apical growth rates, cell size and root growth after IAA treatment in wild-type and transgenic *Arabidopsis* lines

(a) Plant line		Root apical growth		n
		Rate (μ m h ⁻¹)	Per cent of wild type	
Col-0	(–)	254.1	100	57
3A	(+)	341.4*	134.4	20
3A	(–)	259.4	102.1	30
4A3	(+)	291.6*	114.8	47
6A	(+)	354.1*	139.5	20
6A	(–)	252.4	99.3	16
7A	(+)	344.9*	135.7	24
7A	(–)	249.8	98.3	19
8A	(+)	335.4*	131.9	21
8A	(–)	258.6	101.8	31
11A1	(+)	258.8	101.8	45
2A5	(+)	253.1	99.6	56

(b) Cell type	Col-0 (wild type)		Plant line 7A (transgenic)		8A (transgenic)	
	Size (μ m)	n	Size (μ m)	n	Size (μ m)	n
Epidermis	137	37	129	34	158	12
Cortex	159	31	135*	7	160	9
Endodermis	109	23	90*	22	107	11
Pericycle	73	26	67	19	71	57

(c) Plant line	Growth of seedling root system					
	Fresh weight (mg)		Dry weight (mg)		DNA per root (μ g)	
	3 d	6 d	3 d	6 d	3 d	6 d
Col-0	11	25	1.7	2.4	5	14
4A3	31	136	4.5	15.4	8	35
6A	30	155	4.2	19.3	10	46
7A	24	156	3.5	16.8	9	38
8A	18	134	2.5	12.8	8	33

Lines 3A, 6A, 7A, 8A are heterozygous T1 populations with more than one introduced transgene; (+) denotes plants with increased *cyc1At* transcript, (–) plants with wild-type levels of *cyc1At* transcripts. The following T2 lines are homozygous for *cdc2aAt::cyc1At*: 2A5, 4A3 and 11A1; constitutive *cyc1At* expression in 4A3, but not in 2A5 and 11A1, exceeds that induced by IAA in wild type (Fig. 1). n, Number of plants analysed; asterisk indicates a significant difference from the wild type; for a, $P < 0.001$, for b, $P < 0.01$. Homozygous or heterozygous seed were plated on MS agar and plants grown in vertical orientation for 7 d with a 16 h day/8 h night schedule at 22 °C. Four images of each plate were acquired with a Speedlight Platinum frame grabber (Lighttools Research) at 24-h intervals and root growth analysed with NIH-Image by measuring the displacement of root apices. After growth analysis, roots from 10 plants of each class were collected and their RNA analysed (not shown). To measure cell sizes, roots were cleared by overnight incubation in saturated chloral hydrate, visualized with Nomarski optics, photographed and analysed with NIH-Image. Statistical analysis (t-test with unpaired variances) was performed with MS Excel. Root growth in IAA-treated plants was assessed 3 and 6 d after induction by determining the fresh wet weight of roots excised from liquid-grown plants and then the dry weight after lyophilization for 24 h. Total DNA was extracted from dried material²⁸.

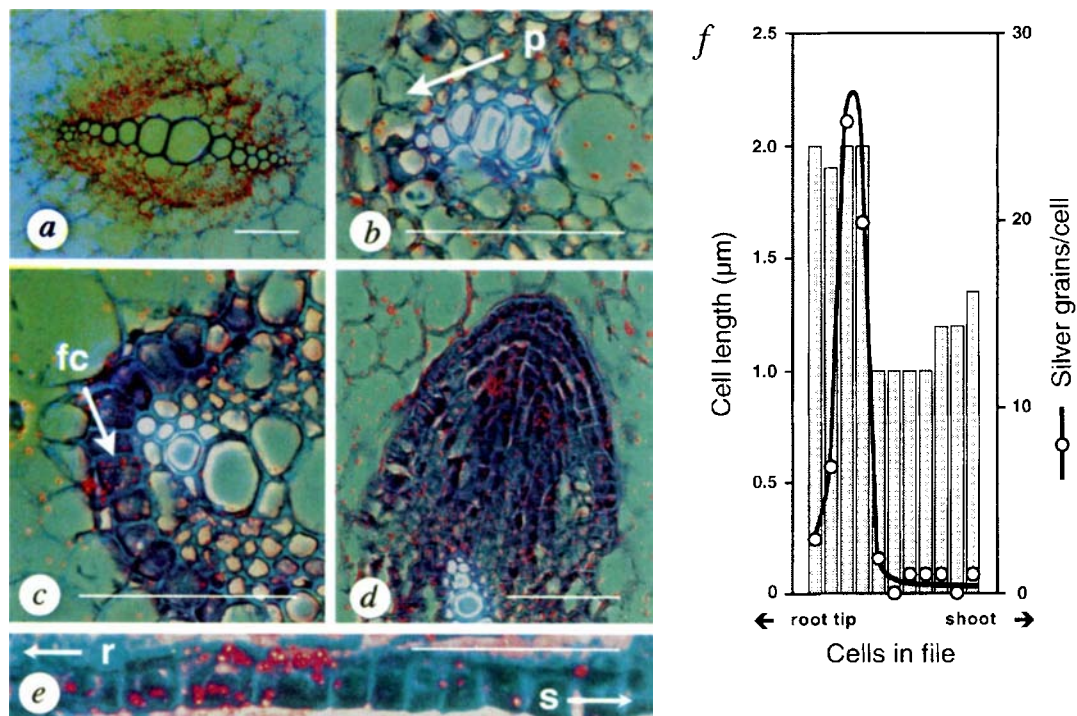
initiation of lateral roots, together with the pattern of *cyc1At* promoter activity deduced from the expression of *cyc1At::uidA* gene fusions in transgenic *Arabidopsis*⁹, suggested that cyclin abundance might be a key factor in regulating root growth and development. To test this idea we generated transgenic *Arabidopsis*¹⁰ containing *cyc1At* under the control of the *cdc2aAt* promoter and obtained five transformants in which the level of *cyc1At* mRNA in untreated roots exceeded that in IAA-stimulated roots of wild-type plants (Fig. 1c). Investigation of these lines showed that strong expression of the *cdc2aAt::cyc1At* transgene caused a marked increase in the rate of organized root growth (Fig. 3a). In heterozygous T2 progeny, increased growth rate, measured by displacement of the apex of the main root in time-lapse photography, strictly co-segregated with transgene expression; individuals lacking the transgene grew at the same rate as wild-type plants (Table 1a). The average size of epidermal, cortical, endodermal and pericycle cells was equivalent or slightly reduced in *cdc2aAt::cyc1At* transformants compared to wild-type plants (Table 1b), and hence increased growth reflects increased cell number rather than cell size. The pattern of spontaneous lateral root initiation and overall root morphology were indistinguishable in wild-type and transgenic plants (Fig. 3a). When treated with 1 μ M IAA, which induces well separated lateral root primordia, the frequency of primordia initiated per unit length of the main root was not altered (mean, 1.08 initials mm^{-1} , with a standard deviation of 0.09 in wild type, compared with 1.14 ± 0.07 and 1.09 ± 0.13 , respectively, in the two transgenic lines). Growth and development of lateral roots following induction by 10 μ M IAA, however, was markedly accelerated in the *cdc2aAt::cyc1At* transformants, giving rise to a much-enlarged root system (Fig. 3b). Enhanced root growth in *cdc2aAt::cyc1At* plants following IAA treatment superficially resembles the *alf1* phenotype¹¹ these plants have increased amounts of *cyc1At* transcripts (not shown), but in contrast to *cdc2aAt::cyc1At* transformants, *alf1* plants initiate supernumerary lateral roots. The several-fold greater

gain of fresh weight in IAA-treated *cdc2aAt::cyc1At* plants compared to equivalent wild-type controls was accompanied by marked increases in DNA content and dry weight (Table 1c). Confocal microscopy confirmed that the enhanced growth response to IAA, also seen in several lines with weaker *cdc2aAt::cyc1At* expression, did not reflect transgene stimulation of cell vacuolation or elongation. Ectopic cyclin expression thus enhances root growth by stimulation of cell division in meristems, increasing the rate of cell production without altering meristem organization, but it is not known whether these effects are mediated by interaction with *cdc2a* or with other cyclin-dependent kinases².

Our data indicate that *cdc2aAt::cyc1At* expression is sufficient to enhance growth from established root apical meristems, suggesting that the cell cycle regulates meristem activity. But failure to induce gratuitous lateral root primordia by ectopic expression of *cyc1At* under the control of the *cdc2aAt* promoter, which gives strong expression in quiescent pericycle cells^{4,12}, indicates that there are additional control points in the generation of a new apical meristem—either through post-translational regulation of cyclin-dependent protein kinase activity or the operation of parallel regulatory pathways. In most animal cells, commitment to cell division occurs late in G1 (ref. 13), and cyclin D1 and cyclin E are rate-limiting for progression through G1 in cultured cells^{14–16}. Increased cyclin D1 is found in several tumours^{17–19}, and ectopic expression in transgenic mice promotes hyperplasia and adenocarcinomas²⁰. In contrast, ectopic expression of *cyc1At* does not result in neoplasia but stimulates organized growth without altering meristem organization or size, as monitored by confocal microscopy. Root morphology was not altered in our transgenic plants and increased growth was accompanied by accelerated development of the root system. Cyclin expression is thus a limiting upstream factor in an intrinsic regulatory hierarchy governing meristem activity, organized growth and indeterminate development of the root system. This regulatory hierarchy is

FIG. 2 *In situ* hybridization analysis of *cdc2aAt* and *cyc1At* transcripts in root apices and developing lateral roots. a–d, Cross sections of quiescent roots (a, b) or proliferating cells in primordia (c, d) were hybridized to *cdc2aAt* (a) or *cyc1At* (b–d) anti-sense probes. e, f, *cyc1At* mRNA abundance in contiguous meristematic cell files in root apices. Transcript accumulation is indicated by silver-grain deposition and visualized by indirect red illumination. Scale bar is 10 μ m in a–d, 5 μ m in e. fc, Founder cell accumulating *cyc1At* transcripts; p, pericycle cell layer; r, towards the root apex; s, towards the shoot. METHODS. Tissue samples for *in situ* hybridization were treated with 10 μ M IAA (b–d; as for Fig. 1). After 8 or 24 h incubation, radish (*Raphanus sativa* var Scarlet Globe) roots were processed as described²⁸.

Sections (8 μ m) were hybridized to a ³³P-labelled RNA probe, corresponding to nt 674–1,004 of *cyc1At*⁶ (b–e) or to a ³⁵S-labelled probe, used in a, corresponding to nt 661–1,386 of *cdc2aAt*²⁶, for 14 h at 48 °C in 50% formamide. After hybridization, final washes were for 1 h at 58 °C in 0.015 M NaCl; slides were then exposed for 3 weeks (*cyc1At*) or 5 days



(*cdc2aAt*). After developing, silver grains were illuminated laterally with red light, specimens were visualized by phase contrast and double exposures were taken on FUJI Velvia film. Images were assembled in ADOBE Photoshop. For the analysis summarized in f, silver grains were counted and cell size measured in the cell file shown in e.

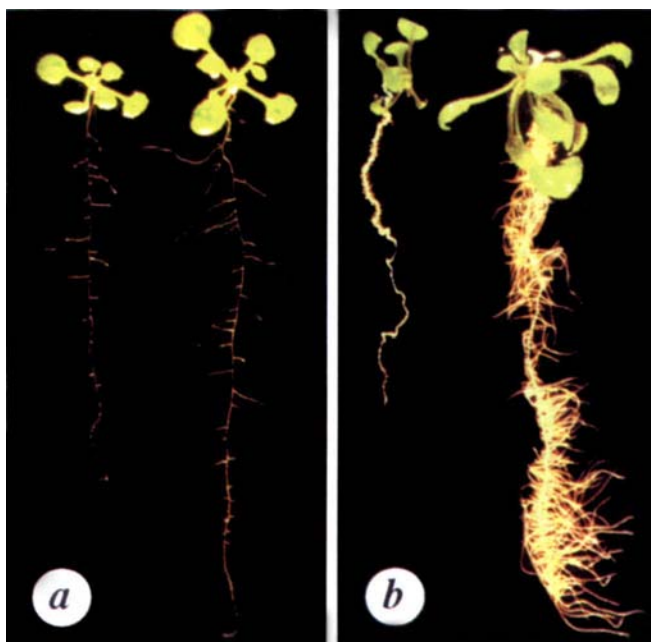


FIG. 3 Increased root-growth rate in *Arabidopsis* ectopically expressing *cyc1At* cyclin. **a**, Wild-type (left) or transgenic line 6A (T1 generation) containing the *cdc2aAt::cyc1At* gene fusion (right). *Arabidopsis* seeds were plated on MS (3% sucrose) agar and grown in a vertical orientation for 7 d. Plants transformed with vector alone or with unrelated promoter:*uidA* constructs or with a *cdc2aAt::cyc1At* fusion, in which the *cdc2aAt* 5' untranslated leader was interrupted by a DS transposon insertion, did not show this phenotype. **b**, Wild-type (left) or transgenic line 6A (T1 generation) (right) 6 d after IAA induction of lateral roots. One-week-old seedlings grown hydroponically were treated with 10 μ M IAA to stimulate lateral root development.

METHODS. An *NheI* site was introduced in the third codon of the *cyc1At* cDNA by *in vitro* mutagenesis and this open reading frame ligated to the *cdc2aAt* promoter with an *in vitro*-generated *XbaI* site at codon 3. This fragment was ligated into pBiB-Hyg⁹ and transfected into *Agrobacterium tumefaciens* GV3101³⁰. *Arabidopsis* (ecotype Columbia) was transformed by vacuum infiltration¹⁰, and transgenic seedlings (T0 generation) were selected on MS plates containing 30 μ g ml⁻¹ hygromycin. 52 independent transgenic lines were obtained and elevated levels of *cyc1At* mRNA were detected in 9 of the 11 lines analysed. Growth assays were done on heterozygous T1 and homozygous T2 progeny as indicated.

different from that in animals, in which determinate development limits proliferative growth, and is illustrated by the strict morphogenetic control of cell division that occurs during muscle differentiation^{21,22}. It may underlie the striking plasticity of root growth and development²³. We propose that cyclin abundance functions as a 'rheostat' to allow flexible growth control in response to changes in the environment such as nutrient availability. □

Received 3 October 1995; accepted 31 January 1996.

1. Steeves, T. A. & Sussex, I. M. *Patterns in Plant Development* 1–388 (Press Syndicate of the University of Cambridge, UK, 1989).
2. Murray, A. & Hunt, T. *The Cell Cycle* (Freeman, New York, 1993).
3. Martinez, M. C., Jorgensen, J.-E., Lawton, M. A., Lamb, C. J. & Doerner, P. W. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7360–7364 (1992).
4. Blakely, L. M. & Evans, T. A. *Plant Sci. Lett.* **14**, 79–83 (1979).
5. Hemerly, A. et al. *EMBO J.* **14**, 3295–3299 (1995).
6. Hemerly, A., Bergounioux, C., Van Montagu, M., Inzé, D. & Ferreira, P. *Proc. natn. Acad. Sci. U.S.A.* **89**, 3295–3299 (1992).
7. Dolan, L. et al. *Development* **119**, 71–84 (1993).
8. Fobert, P. R., Coen, E. S., Murphy, G. J. P. & Doonan, J. H. *EMBO J.* **13**, 616–624 (1994).
9. Ferreira, P. C. G. et al. *Plant Cell* **6**, 1763–1774 (1994).
10. Bechtold, N., Ellis, J. & Pelletier, G. C. R. *Acad. Sci. Paris, Life Sci.* **316**, 1194–1199 (1993).
11. Celenza, J. L., Grisafi, P. L. & Fink, G. R. *Genes Dev.* **9**, 2131–2142 (1995).
12. Hemerly, A. S. et al. *Plant Cell* **5**, 1711–1723 (1993).
13. Pardee, A. B. *Science* **246**, 603–608 (1989).
14. Ohtsubo, M. & Roberts, J. M. *Science* **259**, 1908–1912 (1993).
15. Quelle, D. E. et al. *Genes Dev.* **7**, 1559–1571 (1993).
16. Resnitsky, D. & Reed, S. I. *Molec. cell. Biol.* **15**, 3463–3469 (1995).

17. Motokura, T. et al. *Nature* **350**, 512–515 (1991).
18. Rosenberg, C. L. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 9638–9642 (1991).
19. Withers, D. A. et al. *Molec. cell. Biol.* **11**, 4846–4853 (1991).
20. Wang, T. C. et al. *Nature* **369**, 669–671 (1994).
21. Halevy, O. et al. *Science* **267**, 1018–1021 (1995).
22. Skapek, S. X., Rhee, J., Spicer, D. B. & Lassar, A. B. *Science* **267**, 1022–1024 (1995).
23. Drew, M. C. *New Phyt.* **75**, 479–490 (1975).
24. Murashige, T. & Skoog, F. *Physiol. Plant.* **15**, 473–497 (1962).
25. Ausubel, F. M. et al. *Current Protocols in Molecular Biology* (Greene and Wiley-Interscience, New York, 1987).
26. Hirayama, T., Imajuku, Y., Anai, T., Matsui, M. & Oka, A. *Gene* **105**, 159–165 (1991).
27. Norris, S. R., Meyer, S. E. & Callis, J. *Plant molec. Biol.* **21**, 895–906 (1993).
28. Drews, G. N., Bowman, J. L. & Meyerowitz, E. M. *Cell* **65**, 991–1002 (1991).
29. Becker, D., Kemper, E., Schell, J. & Masterson, R. *Pl. Molec. Biol.* **20**, 1195–1197 (1992).
30. Koncz, C. & Schell, J. *Molec. gen. Genet.* **204**, 383–396 (1986).

ACKNOWLEDGEMENTS. We thank J. Celenza for the *cdc2aAt* promoter, C. Hoeger for peptide synthesis, and J. Chory, A. Colón-Carmona, S. Hedrick, T. Hunter, R. Pennell, V. Vitart and D. Weigel for discussion. This work was supported by grants from the Samuel Roberts Noble Foundation to C.L. and the NRI Competitive Grants Program of the US Department of Agriculture to P.D.; J.-E.J. and J.S. were supported by Fellowships from the Carlsberg Foundation and EMBO, respectively.

Global plasticity in adult visual cortex following reversal of visual input

Yoichi Sugita

Laboratory for Neural Systems, Toyohashi University of Technology, Toyohashi, 441 Japan

THE reversal or displacement of the retinal image by prism spectacles leads to extreme disruption of visually guided behaviour, but after an extended period of visual transformation normal behaviour is gradually restored. It is unclear whether this adaptation involves a change in visual perception^{1–5}, the learning of new motor responses⁶, a modification of the sensorimotor control system⁷ or a proprioceptive change in the perceived positions of the body parts⁸. Here I describe the effects of visual field reversal on neuronal activity in the monkey visual cortex. After a few months of wearing reversing spectacles, some cells in the primary visual cortex began to respond to stimuli presented not only in the contralateral visual field but also in the ipsilateral field. These cells were not selective for orientation or direction of motion, but responded well to a light flash. This result suggests that adaptation to visual field reversal is mediated, at least in part, by a large-scale functional reorganization at an early stage in the visual processing pathway.

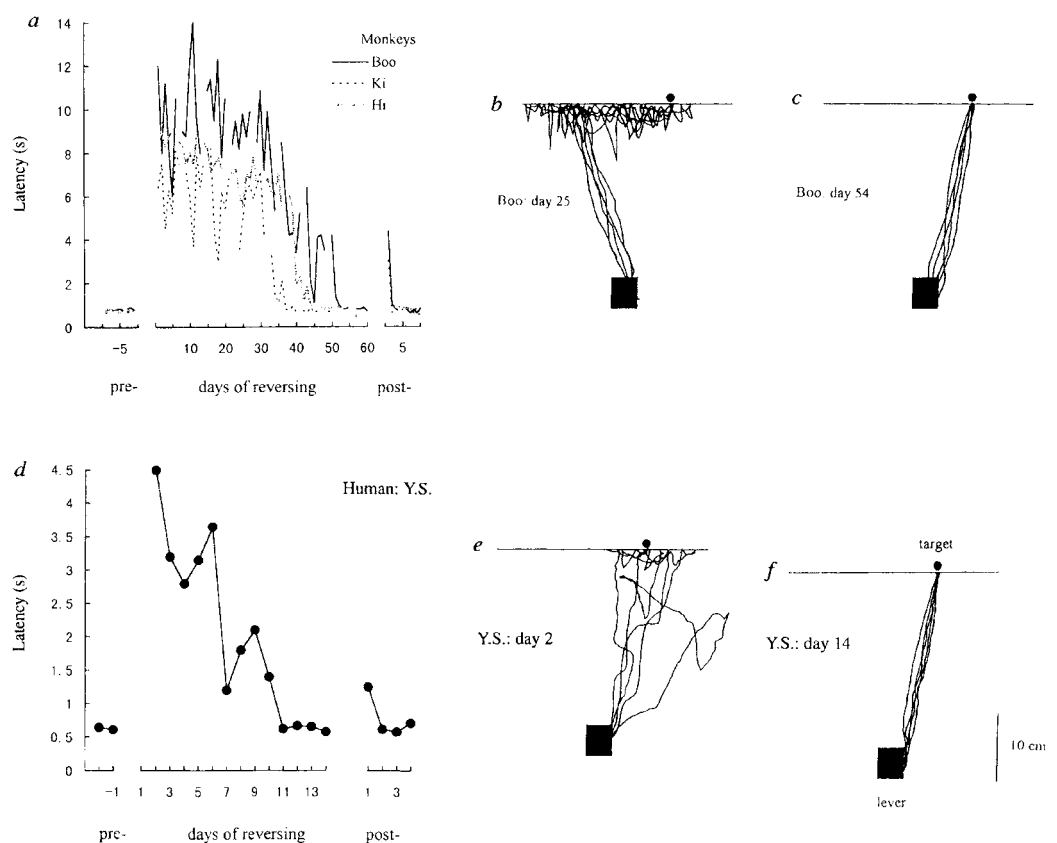
Two male (weighing 4.5 and 5.8 kg, respectively) and one female (4.8 kg) Japanese monkeys (*Macaca fuscata*) wore a hand-made goggle continuously for about 4 months. For the first month no optical distortion was introduced to allow the animals to become accustomed to the restriction of the visual field. The monocular field of view through each eye was about 26° horizontally and 30° vertically, and the binocular field of view was about 40° horizontally and 30° vertically. After the first month, two Dove prisms for achieving left–right reversed vision were mounted in the goggles. Prism axes were adjusted to eliminate diplopia at a distance of 1 m.

The disruption of visually guided behaviour was much more severe than expected from human studies. The animals could not even move by themselves for the first 2 weeks. Food intake also decreased remarkably, presumably as a result of the nausea caused by the swinging of the visual scene. Only after 3 or 4 weeks did they begin to move by themselves and their body weight recovered to the normal level.

The adaptation level was estimated by measuring response latencies for a visually guided reaching behaviour. The behavioural task was to touch a red circle (2 cm in diameter) by hand that was presented on a CRT display. The response latencies

FIG. 1 Response latencies and trajectories of the tip of the middle finger during visually guided reaching behaviour. Human data are also presented for comparison. *a*, Daily changes in latencies for the three animals. *b*, Examples of trajectories of one animal's (Boo) middle finger measured 25 days after the reversal of the retinal image. *c*, Examples of trajectories of the same animal's middle finger measured 54 days after the reversal. *d–f*, Corresponding data obtained from a human subject (Y.S.).

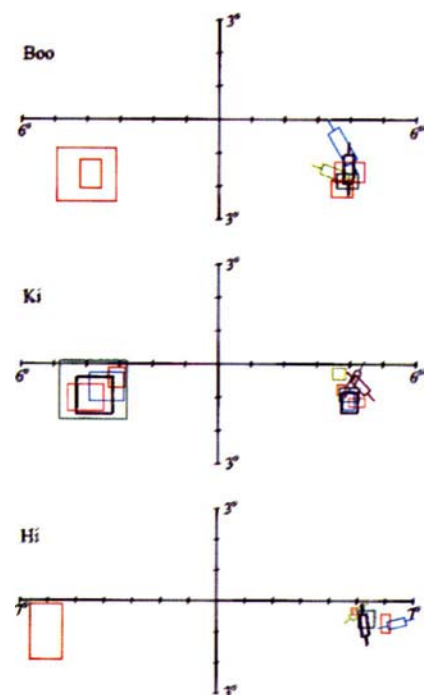
METHODS. The human subject wore the reversing spectacles continuously for 14 days. The same apparatus was used both for animal and human studies except for the chair. A trial began by a subject holding a lever. When the subject kept holding a lever for 2 s, a red circle (2 cm in diameter) was presented on a CRT display. The distance between the lever and the lower edge of the display was 25 cm. The location of the circle was changed from trial to trial. When the subject touched the display at the location where the red circle was presented, the circle was erased. The human subject was instructed to touch the display as quickly as possible. The animal was given a few drops of orange juice when it correctly touched the display. A video camera was used for off-line analysis of the trajectories of hand movement. Although the animals and the human subject could use both hands, two animals (Boo and Hi) and the human



subject always used their right hand. One animal (Ki) preferred the left hand. While the animals were wearing the goggles, they performed the task every day apart from the day when the physiological experiment was done. Fifty trials were made in each session. The animals could eat freely in their home cage but water was available only for an hour after the daily experiment.

FIG. 2 Receptive fields of V1 cells in the left hemisphere measured in the last session of the reversing period. In the session, three penetrations were made for monkeys Boo and Ki, and four penetrations for monkey Hi. Squares with bars represent the receptive fields of orientation cells and squares without bars those of non-orientation cells. The contra- and ipsilateral receptive fields of the bilateral cells are shown in the same colour.

METHODS. The general methods of preparation and recording were similar to those described previously^{13,14}. Three Japanese monkeys were chronically prepared for repeated recordings. The recording session was started with an induction of anaesthesia with ketamine hydrochloride (10 mg kg⁻¹ i.m.) and the goggles were removed. Throughout the recording the animal was immobilized with gallamine triethiodide and anaesthesia was maintained by artificial ventilation with a gas mixture of N₂O/O₂. A glass-coated Elgiloy electrode (2.5–3.5 MΩ at 1 kHz) was inserted into the left hemisphere for extracellular recordings. The electrode was penetrated vertically to the surface of dura. In the later sessions, when the first isolated cell had the orientation preference, the electrode was retracted and another penetration was made. A light spot (36 cd m⁻²) was projected on a translucent tangent screen from the side opposite the animal. The size of the spot was 0.25, 0.5, 1 or 2° in diameter. First the preferred eye and stimulus size were determined. However, when the receptive field was close to the midline, a smaller size of stimulus was used so that another visual hemi-field would never be stimulated. The location and extent of the receptive field was determined for excitatory responses using a manually controlled projector. Horizontal edges were first determined and vertical edges were decided thereafter. However, the receptive fields were measured in spherical polar coordinates¹⁵ and the area of the fields was calculated assuming that they were elliptical. The experiments were done under dim illumination so that the luminance of the screen was 3.2 cd m⁻².



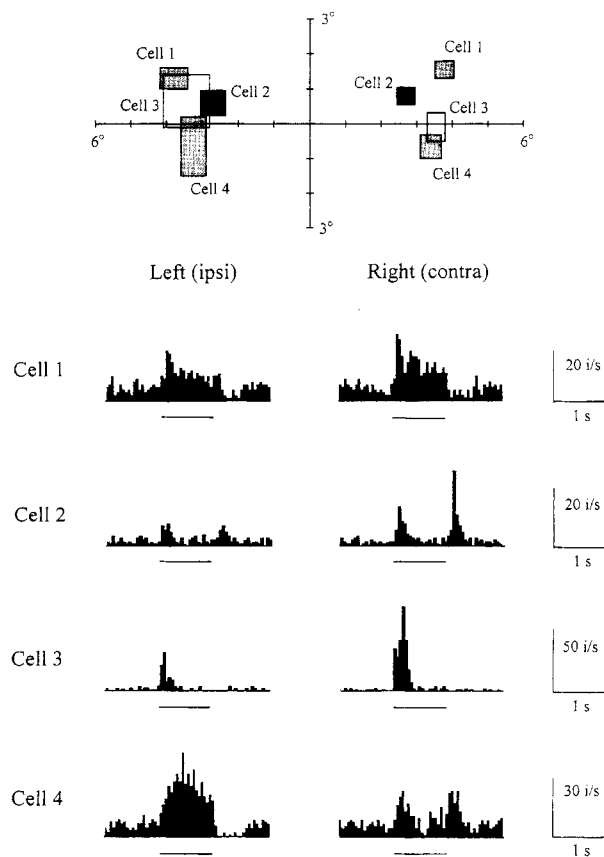


FIG. 3 Receptive fields of four V1 cells in the left hemisphere and peristimulus time histograms (PSTHs) for a light spot of 1 s duration. The stimulus was presented to the left eye for each cell. The underlined section below each histogram represents the period of stimulation. PSTHs of the responses for the preferred stimulus size were made on the basis of 7 trials. However, when the preferred size was larger than the receptive field, the smaller light spot was used. I/s, impulses per second.

increased abruptly when the retinal image was reversed. About a month after reversing the retinal image, the latencies began to decrease and came to baseline level at about 1½ months after reversing. The after-effect was also examined when the Dove prisms were removed from the goggles. As with the human data^{1-3,5}, the effect disappeared very quickly, only being observed on the day the prisms were removed (Fig. 1).

During single-unit recording, the goggles were removed and the animal was anaesthetized and immobilized. Of special interest was the fact that some V1 cells began to respond to stimuli presented in the ipsilateral visual field (Fig. 2). These dramatic changes were observed about 1½ months after wearing left-right reversing spectacles. A total of 119 V1 cells were isolated. Out of 119 cells, 43 were found to respond to ipsilateral as well as contralateral stimulation. These 43 cells apparently had no preference to the orientation or to the direction of motion. Of these 43 cells, 32 were examined quantitatively.

The cells with bilateral receptive field responded both to the ipsilateral and contralateral stimulations in a similar way (Fig. 3). All of them responded well to light flash; 13 showed sustained response during the stimulus period; 10 responded transiently to the onset of the stimulus; and 8 responded transiently to the onset and offset of the stimulus. Only one exceptional cell responded transiently both to the onset and the offset of the stimulus presented in the contralateral receptive field, but showed sustained response to the ipsilateral stimulations.

Newly formed ipsilateral receptive fields were located bisymmetrically to the original contralateral receptive fields: the distance from the centre of the ipsilateral receptive field to the vertical midline was almost equal to that for the contralateral receptive field (Fig. 4a). The sizes of the ipsilateral receptive fields were bigger than those of the contralateral receptive fields (Fig. 4b), although the cells tended to respond more vigorously to the contralateral stimulation (Fig. 4c).

In addition to the above-mentioned 43 cells, 12 non-orientation cells were isolated. However, these 12 cells did not respond to the ipsilateral stimulation. All of these cells showed strong eye preferences: 11 out of 12 cells responded only to stimuli presented to one of two eyes but not to the other eye. On the other hand, 35 out of 43 that had the ipsilateral receptive field were classified as binocular cells. A total of 64 orientation cells was also tested. Out of 64 cells, 21 were classified as simple cells and the remaining 43 cells as complex cells. None of these cells responded to the ipsilateral stimulation.

About a month after the first observation of the response to ipsilateral stimulations, the Dove prisms were removed from the

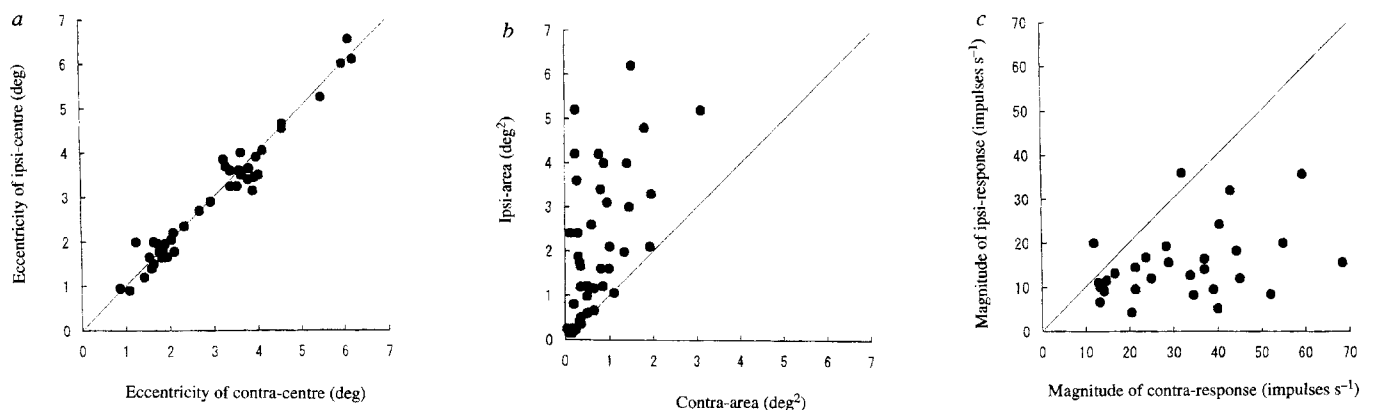


FIG. 4 Difference between ipsi- and contralateral receptive fields for individual cells. *a*, Eccentricity of the ipsilateral receptive field is plotted against that of the contralateral receptive field. *b*, Size of the ipsilateral receptive field is plotted against that of the contralateral receptive field. *c*, Magnitude of response to ipsilateral stimulations was plotted against the

response to contralateral stimulations. The magnitude was quantified by calculating the average firing rate during 7 repetitions of the stimulus cycle and subtracting the average spontaneous firing rate immediately before stimulation.

goggles. Single-unit recordings were resumed for three monkeys at 2, 5 and 7 days after removing the prisms, respectively. Three recording sessions were given to each animal after removing the prisms and 38 non-orientation cells were isolated. However, none of these cells was found to respond to ipsilateral stimulations, although 31 out of 38 cells were classified as binocular cells. From these observations it is clear that the response to the ipsilateral stimulation is strongly related to the adaptation to optical distortion.

The activity of the cells with bilateral receptive fields might be correlated with the so-called 'piecemeal adaptation' to optical distortions, described well in human studies³: after about a week of adaptation, a part and some attributes of the visual scene appeared normal whereas others remained in the left-right reversed form. Such an apparent change in visual perception requires at least several days of adaptation¹⁻⁵, whereas an appropriate visuomotor response could be learned within minutes^{7,8}. Correspondingly, the response of V1 cells to the ipsilateral stimulation was observed only after visuomotor adaptation had been completed.

The cells with bilateral receptive fields would receive inputs from higher cortical areas that have strong interhemispheric connections because cortical visual areas are organized in a reciprocal fashion, that is, an area not only sends signals to but also receives them from another area⁹. The interhemispheric connections have already been demonstrated in the secondary visual cortex, as well as in a part of the primary visual cortex^{10,11}.

Furthermore, the connections were observed at regions of high cytochrome oxidase activity¹⁰, the cells of which show no preference either to the orientation or the direction of motion. It is therefore very likely that the cells with bilateral receptive fields receive signals from the cells of regions rich in cytochrome oxidase.

It was already known that the receptive field structure changes after the removal of visual input¹². This study, however, demonstrates that these changes are not restricted to local regions but may take place in the whole area of the primary visual cortex. □

Received 20 November 1995; accepted 6 February 1996.

1. Stratton, G. M. *Psychol. Rev.* **4**, 341-360; 463-481 (1897).
2. Taylor, J. G. *The Behavioral Basis of Perception* (Yale Univ. Press, New Haven, 1962).
3. Köhler, I. *Psychol. Issues* **3**, 1-173 (1964).
4. Shimojo, S. & Nakajima, Y. *Perception* **10**, 391-402 (1981).
5. Sugita, Y. *Percept. Motor Skills* **79**, 1047-1054 (1994).
6. Day, R. H. & Singer, G. *Psychol. Bull.* **67**, 307-322 (1967).
7. Held, R. & Freedman, S. J. *Science* **142**, 455-462 (1963).
8. Harris, C. S. *Science* **140**, 812-813 (1962).
9. van Essen, D. C. & Maunsell, J. H. R. *Trends Neurosci.* **6**, 370-375 (1983).
10. Cusick, C. G., Gould, H. J. III & Kaas, J. H. J. *comp. Neurol.* **230**, 311-336 (1984).
11. Spatz, W. B. & Kunz, B. *Neurosci. Lett.* **48**, 49-53 (1984).
12. Gilbert, C. D. & Wiesel, T. N. *Nature* **356**, 150-152 (1992).
13. Sugita, Y. & Tanaka, K. *Neuroreport* **2**, 751-754 (1991).
14. Tanaka, K., Sugita, Y., Moriya, M. & Saito, H. J. *Neurophysiol.* **69**, 128-142 (1993).
15. Bishop, P. O., Kozak, W. & Vakkur, G. J. *J. Physiol., Lond.* **163**, 466-502 (1962).

ACKNOWLEDGEMENTS. This work was supported by grants from the Ministry of Education and Culture, Japan and H. Nakayama science promotion foundation.

Activation of the primary visual cortex by Braille reading in blind subjects

Norihiro Sadato*†, Alvaro Pascual-Leone*, Jordan Grafman‡, Vicente Ibañez*, Marie-Pierre Deiber*, George Dold§ & Mark Hallett*

* Human Motor Control Section, and ‡ Cognitive Neuroscience Section, Medical Neurology Branch, and § Research Service Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892-1428, USA

† Biomedical Imaging Research Center, Fukui Medical School, 23 Shimoaizuki, Matuoka, Fukui 910-11, Japan

PRIMARY visual cortex receives visual input from the eyes through the lateral geniculate nuclei, but is not known to receive input from other sensory modalities¹. Its level of activity, both at rest and during auditory or tactile tasks, is higher in blind subjects than in normal controls², suggesting that it can subserve non-visual functions; however, a direct effect of non-visual tasks on activation has not been demonstrated²⁻⁴. To determine whether the visual cortex receives input from the somatosensory system⁵⁻⁸, we used positron emission tomography (PET) to measure activation during tactile discrimination tasks in normal subjects and in Braille readers blinded in early life. Blind subjects showed activation of primary and secondary visual cortical areas during tactile tasks, whereas normal controls showed deactivation. A simple tactile stimulus that did not require discrimination produced no activation of visual areas in either group. Thus, in blind subjects, cortical areas normally reserved for vision may be activated by other sensory modalities.

Eight proficient Braille readers were studied during Braille reading (Table 1). Eight-character, non-contracted Braille-letter strings were presented every 2.4 seconds. In the 'word' condition, 41 words and 3 non-words were presented. Subjects were asked to

utter 'num' only when they encountered the non-words. In the 'non-word' condition, all were non-words except for three words. Subjects were told to respond only to the words. To compare the behaviour of the posterior brain regions of blind and normally sighted individuals, non-Braille tactile tasks were performed by six other blind and ten sighted subjects (Table 1). One non-discrimination task ('sweep') and three discrimination tasks ('angle', 'width' and 'character') were included. Stimulus patterns were presented every 5 seconds. In the 'sweep' task, subjects were to sweep their index finger over a rough surface homogeneously covered with Braille dots, without response. In the 'angle' task, two grooves with the same width (3.2 mm, separated by 6.4 mm) cut in a piece of paper homogeneously covered by Braille dots were presented in pairs. Subjects were to respond ('num') when the angles were the same. In the 'width' task, a response was expected when the two vertical grooves (either 3.2 mm or 4.6 mm in width) presented had the same width. In the 'character' task, three upper-case English letters embossed with Braille dots (12.7 mm in height and 10.2 mm in width) were presented together. Subjects were to respond if the three letters were identical. In each task, the pattern was identical in 3 of 30 presentations. No spatial or mental imagery was requested.

Braille reading by the blind activated the medial occipital lobe (Brodmann's area 17) extending to the extrastriate cortices bilaterally (Fig. 1a, Table 2). The three non-Braille discrimination tasks also activated the primary visual cortex in the blind subjects, (Fig. 1b), but with a smaller increase of regional cerebral blood flow (rCBF) than Braille reading (Table 2), whereas in sighted subjects these tasks elicited a decrease in rCBF (Fig. 1c). The non-discrimination task did not activate the primary visual cortex in either the blind or the sighted subjects (Table 2).

Whereas the non-discrimination task did not activate the primary visual cortex of the blind subjects, the tactile discrimination tasks did. Subjects made verbal responses in the discrimination tasks, but not in the non-discrimination task; however, as the number of responses was very low, it is unlikely that any activation was related to them. Although the tasks used do not allow us to interpret exactly what occurs in the visual cortex, it entails the use of the somatosensory information.

In contrast with the blind, the sighted subjects had a decrease in

rCBF in the primary visual cortex during the tactile discrimination tasks. This suppression could be a by-product of selective attention to the tactile modality. A decrease in rCBF in the auditory and somatosensory cortices during visual tasks has been reported, and it has been suggested that selective attention to one sensory modality is associated with decreased activity in areas dedicated to processing input from other sensory modalities⁹. Selective attention to vibrotactile stimulation of the fingers in normal subjects increased the CBF response in the primary sensorimotor cortex¹⁰. This evidence suggests that selective attention does not affect the primary visual cortex the same way in blind and sighted subjects.

Visual imagery¹¹ is an unlikely explanation of our findings because subjects blinded early in life have little or no 'visual' memory to aid their Braille reading, which was usually learned after the loss of sight. Spatial imagery based on haptic ('active touch') experience exists in congenitally blind people¹². The congenitally blind subjects who performed Braille-reading tasks showed the same activation of the primary visual cortex as the other blind subjects.

The greater activation in the primary visual cortex during Braille reading than during non-Braille discrimination tasks could be a result of many factors, such as faster presentation of the stimuli, increased complexity of the task, or lexical processing.

TABLE 1 Subject data and task performance

	Braille (N = 8)	Blind non-Braille (N = 8)	Sighted non-Braille (N = 10)
Age (years)	49.6 ± 7.1	42.2 ± 7.7	32.4 ± 8.1
Sex (men/women)	4/4	4/2	3/7
Handedness	right	7 right, 1 left	right
Age of onset of blindness	4.3 ± 5.5	1.5 ± 2.1	
Cause of blindness (congenital/acquired)	2/6	1/5	
Age of learning Braille	5.9 ± 1.1	5.3 ± 0.8	
Years of reading Braille	43.8 ± 6.6	36.8 ± 7.6	
Daily practice (h per day)	2.2 ± 1.1	2.8 ± 1.9	
Overall performance (% accuracy)*	95.7 ± 3.2	92.5 ± 7.9	87.1 ± 6.8
Non-word task performance (% accuracy)*	97.1 ± 2.7		
Word task performance (% accuracy)*	94.4 ± 4.0		

* Accuracy = (correct responses/number of presentations) × 100.

FIG. 1 a, Adjusted mean rCBF in blind subjects reading Braille with the right index finger compared with rest. A statistical parametric map of group analysis in three orthogonal sections was superimposed on a typical anatomical magnetic resonance image (MRI) unrelated to the study's subjects (as shown in b and c). All PET scans were normalized into the standard, proportional stereotaxic space of Talairach and Tournoux¹⁹. The red lines indicate the projections of each section that cross in the centre of the activation in the primary visual cortex; the Talairach's coordinates are: $x = -16$ mm, $y = -98$ mm, and $z = -8$ mm. The coronal section is 98 mm posterior to the anterior commissure, the transverse section is 8 mm below the anterior-posterior commissural line, and the sagittal section is 16 mm left of the mid-sagittal plane. Only pixels are shown that were significantly different between conditions at $P < 0.05$ with a correction for multiple comparisons²⁰ to keep the false-positive rate at the defined level for the entire brain. Activation of V1 during Braille reading was also shown by a functional MRI study²¹. b, Activation in blind subjects performing non-Braille discrimination tasks compared with rest. Activation in the primary visual cortex is seen with smaller Z-score than that observed with Braille reading (Table 2). Talairach's coordinates in the centre of the activated area are: $x = -6$ mm, $y = -98$ mm, and $z = -16$ mm. Only pixels that were significantly different between conditions at $P < 0.001$ without a correction for multiple comparisons are shown, as our search was restricted to the primary visual cortex by the results of the Braille-reading task by the blind subjects. c, Adjusted mean rCBF in sighted subjects performing non-Braille discrimination tasks compared with rest. There is a decrease of rCBF in the primary visual cortex. Talairach's coordinates in the centre of the deactivated area are $x = -6$ mm, $y = -98$ mm, and $z = -16$ mm. Only pixels that were significantly different between conditions at $P < 0.001$ without a correction for multiple comparisons are shown.

METHODS. PET scanning was performed with a Scanditronix PC2048-15B (Uppsala, Sweden) 15-slice tomograph with interslice spacing of 6.5 mm. Images were reconstructed to a full width at half maximum of 6.5 mm. Images of cerebral blood flow were obtained by summing the activity occurring during the 60-s period following the initial increase in cerebral radioactivity after an intravenous bolus injection of 30 mCi of ^{15}O -labelled water. Each task began 10 s (Braille task) or 30 s (non-Braille task) before the tracer injection, and continued for the duration of the scan. The order of presentation was counterbalanced. The initial and final scans of a series of 10 were done with the subject at rest. The data were analysed with statistical parametric mapping²²⁻²⁴.

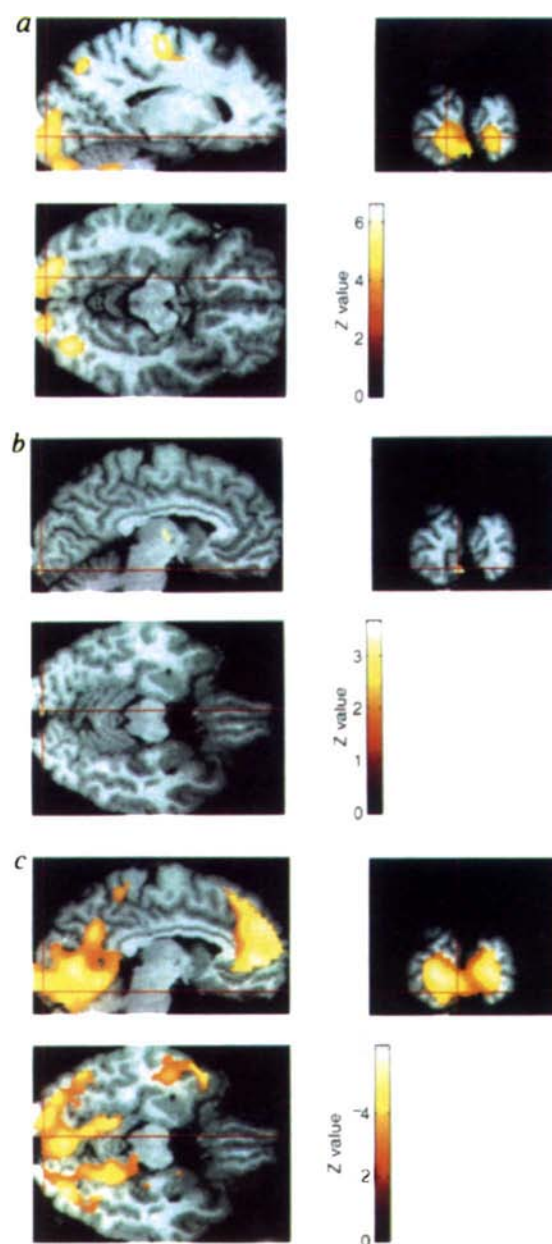


TABLE 2 Activation in the primary visual cortex by tactile discrimination tasks performed by the right index finger

	Non-discrimination (‘sweep’) task versus rest		Non-Braille tactile discrimination tasks versus rest		Braille reading versus rest
	Sighted (n = 10)	Blind (n = 6)	Sighted (n = 10)	Blind (n = 6)	Blind (n = 8)
Change in CBF (%)	-2.71	1.09	-3.87	6.48	9.73
Z-score	-1.98	0.37	-3.34*	3.64†	5.61‡
Talairach's coordinates (ref. 16)	(-6, -98, -16)	(-6, -98, -16)	(-6, -98, -16)	(-6, -98, -16)	(-16, -98, -8)

In the Braille-reading task, activation in the primary visual cortex was similar for reading with the right or left index finger, hence, data are shown only for the right index finger. There was no significant difference between reading words and reading non-words. In the non-Braille tactile discrimination tasks, the data for angle, width and character, compared with the rest condition, were pooled, because the results were similar to those obtained for comparison of the individual conditions. A Bonferroni-type correction was not applied to the results of the non-Braille tactile discrimination tasks because our search was restricted to the primary visual cortex by the results of the Braille reading task by the blind subjects.

* $P < 0.001$ (uncorrected for multiple comparisons).

† $P = 0.0001$ (uncorrected for multiple comparisons).

‡ $P < 0.05$ (with three-dimensional Bonferroni-type correction).

Neuronal mechanisms of cross-modal plasticity, such as unmasking of silent inputs, stabilization of normally transient connections, or axonal sprouting, are based mainly on cross-modal plasticity of neighbouring cortical regions¹³. It has been shown that in cat anterior ectosylvian cortex, where spatial processing is a common function, auditory representation increased in size after visual deprivation¹⁴, which led to the hypothesis that two different inputs to the region were competitive, and that deprivation of one modality accelerated the expansion of the competing pathway. A PET study with sighted subjects¹⁵ showed that a tactile discrimination task activated the parietal cortices, but not the occipital cortex. In the monkey, the posterior parietal association cortex (area 7) is interconnected with the visual association cortex (dorsolateral area 19)¹⁶. Early visual deprivation in the monkey made most neurons in areas 7 and 19 responsive to somatic exploration¹⁷. It is known that diffuse reciprocal projections link area 19 to the primary visual cortex¹⁸. These findings suggest that somatosensory input could be transferred to the primary visual cortex through the visual association areas during Braille reading by blind subjects.

In blind subjects, the primary visual cortex appears capable of reorganizing to accept non-visual sensorimotor information, possibly for further processing. Braille reading, which requires prolonged somatosensory tactile learning, may enhance this plasticity. □

Received 10 August 1995; accepted 8 February 1996.

1. Zeki, S. M. *Nature* **274**, 423–428 (1978).
2. Wanet-Defalque, M. et al. *Brain Res.* **446**, 369–373 (1988).
3. Uhl, F. et al. *Electroenceph. clin. Neurophysiol.* **91**, 249–255 (1994).
4. Uhl, F., Franzen, P., Lindinger, G., Lang, W. & Deecke, L. *Neurosci. Lett.* **124**, 256–259 (1991).
5. Heller, M. A. & Schiff, W. *The Psychology of Touch* (Erlbaum, Hillsdale, 1991).
6. Kennedy, J. M. *Drawing and the Blind: Pictures to Touch* (Yale Univ. Press, New Haven, CT, 1993).
7. Millar, S. *Understanding and Representing Space* (Oxford Univ. Press, New York, 1994).
8. Morgan, M. J. *Molyneux's question: Vision, Touch, and the Philosophy of Perception* (Cambridge Univ. Press, New York, 1977).
9. Haxby, J. et al. *J. Neurosci.* **14**, 6336–6353 (1994).
10. Meyer, E. et al. *Ann. Neurol.* **29**, 440–443 (1991).
11. Le Bihan, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 11802–11805 (1993).
12. Hollins, M. *Understanding Blindness* (Erlbaum, Hillsdale, 1989).
13. Rauschecker, J. P. *Trends Neurosci.* **18**, 36–43 (1995).
14. Rauschecker, J. P. & Korte, M. *J. Neurosci.* **13**, 4538–4548 (1993).
15. Roland, P. E., Eriksson, L., Widen, L. & Stone-Elander, S. *Eur. J. Neurosci.* **1**, 3–18 (1989).
16. Bruce, C., Desimone, R. & Gross, C. G. *J. Neurophysiol.* **46**, 369–384 (1981).
17. Hyvärinen, J., Carlson, Y. & Hyvärinen, L. *Neurosci. Lett.* **26**, 239–243 (1981).
18. Shipp, S. & Zeki, S. *Eur. J. Neurosci.* **1**, 309–332 (1989).
19. Talairach, P. & Tournoux, P. *Co-planar Stereotaxic Atlas of the Human Brain* (Thieme, New York, 1988).
20. Worsley, K. J., Evans, A. C., Marrett, S. & Neelin, P. *J. cereb. Blood Flow Metab.* **12**, 900–918 (1992).
21. Sadato, N. et al. *Neurology* **45**(suppl. 4), A426–A427 (1995).
22. Friston, K. J., Frith, C. D., Liddle, P. F. & Frackowiak, R. S. J. *J. cereb. Blood Flow Metab.* **11**, 690–699 (1991).
23. Friston, K. J., Worsley, K. J., Frackowiak, R. S. J., Mazziotta, J. C. & Evans, A. C. *Hum. Brain Mapp.* **1**, 210–220 (1994).
24. Friston, K. J. et al. *J. cereb. Blood Flow Metab.* **10**, 458–466 (1990).

CORRESPONDENCE and requests for materials should be addressed to M.H. (e-mail: hallett@codon.nih.gov).

Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted *PrP* gene

Suehiro Sakaguchi^{*†}, Shigeru Katamine^{*},
Noriyuki Nishida^{*}, Ryoza Moriuchi^{*},
Kazuto Shigematsu[‡], Tetsuo Sugimoto[§],
Akira Nakatani^{*}, Yasufumi Kataoka^{||},
Takeshi Houtani[§], Susumu Shirabe^{||},
Hitoshi Okada[#], Sumitaka Hasegawa^{*},
Tsutomu Miyamoto^{*} & Tetsuo Noda[#]

Departments of ^{*} Bacteriology, [‡] Pathology, ^{||} Pharmacology, and [¶] Internal Medicine I, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan

[§] Department of Anatomy, Kansai Medical University, Moriguchi 570, Japan

[#] Department of Cell Biology, Cancer Institute, Tokyo 170, Japan

PRION protein (PrP) is a glycoprotein constitutively expressed on the neuronal cell surface. A protease-resistant isoform of prion protein is implicated in the pathogenesis of a series of transmissible spongiform encephalopathies¹. We have developed a line of mice homozygous for a disrupted *PrP* gene in which the whole *PrP*-coding sequence is replaced by a drug-resistant gene². In keeping with previous results^{3–8}, we find that homozygous loss of the *PrP* gene has no deleterious effect on the development of these mice and renders them resistant to prion². The *PrP*-null mice grew normally after birth, but at about 70 weeks of age all began to show progressive symptoms of ataxia. Impaired motor coordination in these ataxic mice was evident in a rotarod test. Pathological examination revealed an extensive loss of Purkinje cells in the vast majority of cerebellar folia, suggesting that PrP plays a role in the long-term survival of Purkinje neurons.

We monitored the biological effect of homozygous disruption of the *PrP* gene in mice by observing 39 homozygous (*PrP*^{-/-}), 90 heterozygous (*PrP*^{+/-}) and 46 wild-type (*PrP*^{+/+}) mice over a long period. The *PrP*^{-/-} mice grew normally and showed no behavioural abnormality for at least one year after birth. But at about 70 weeks old, all the *PrP*^{-/-} mice without exception became easily identifiable by their abnormal gait. The hindquarters of the aged *PrP*^{-/-} mice trembled on initiation of movement and during walking. Analysis of hind footprints revealed that the homozygous mice could not walk along a straight line and that the length of

[†] Present address: Institut für Molekularbiologie I, Universität Zürich, 8093 Zürich, Switzerland.

their steps was shorter than that of $PrP^{+/-}$ or $PrP^{+/+}$ mice (Fig. 1a); these symptoms were progressive. $PrP^{-/-}$ mice older than 90 weeks frequently fell over when they started to walk and were no longer able to get to their feet once they had fallen; most came to show marked kyphosis (Fig. 1b). None of the $PrP^{+/-}$ and $PrP^{+/+}$ mice showed any neurological symptoms, at least up to 95 weeks of age. One exception was a $PrP^{+/-}$ mouse aged 83 weeks, which had flaccid paralysis of the lower limbs, later confirmed as being due to invasion by angiosarcoma of the spinal cord. To evaluate the motor coordination of the mice objectively, we tested the performance of three age groups (73, 83 and 92 weeks old) of each genotype on a rotarod (Fig. 1c). Most (average 72 and 76%, respectively) $PrP^{+/-}$ and $PrP^{+/+}$ mice in all age groups stayed on a rotarod for more than 180 seconds. In contrast, the motor coordination of the oldest group of $PrP^{-/-}$ mice was impaired: 90% of them fell off within 10–20 seconds. About half of the younger $PrP^{-/-}$ mice successfully remained on the rotarod, but the trembling of their hindquarters was accentuated by the effort. All of three 16-week-old $PrP^{-/-}$ mice showed normal motor coordination when compared with age-matched controls (data not shown).

The brains of $PrP^{-/-}$ mice with neurological symptoms were examined for possible pathological changes. The most notable feature was the considerable atrophy of the cerebellum, resulting in a dilatation of the fourth ventricle (Fig. 2). The wet weight of the cerebellar tissue of 83-week old $PrP^{-/-}$ mice was 38.7 ± 1.0 mg (mean $\pm$ s.e.m., $n = 5$), which was significantly lighter than those of age-matched $PrP^{+/-}$ (56.9 ± 2.4 mg; $n = 5$), and $PrP^{+/+}$ (58.4 ± 3.0 mg; $n = 5$), mice ($P < 0.01$ by ANOVA). Characteristic of the histological changes in the cerebellum was an extensive loss of Purkinje cells (Fig. 2c). The molecular layer was obviously thinner, probably as a result of the loss of Purkinje dendritic trees. The Purkinje cells were completely lost or interspersed in the cerebellar folia, although the flocculus, paraflocculus and vermal lobules IX and X preserved a laminar arrangement of these cells (Table 1). Glutamate decarboxylase (GAD) immunostaining confirmed a high degree of disorganization and a reduction in the number of pericellular GABA (γ -aminobutyric acid) synapses of the cerebellar interpositus nucleus and lateral vestibular nucleus (Fig. 3). Haematoxylin–eosin stains of sections of other brain regions examined, including cerebral cortex, hippocampus,

TABLE 1 Cell density of Purkinje cells in cerebellum of 87-week-old mice

Sites in cerebellum	$PrP^{-/-}$	$PrP^{+/-}$	$PrP^{+/+}$
Vermis			
I	$4.0 \pm 0.9^{***}$	21.8 ± 1.8	24.0 ± 0.9
II	$2.3 \pm 1.1^{***}$	22.0 ± 1.1	18.3 ± 2.3
III	$1.8 \pm 0.8^{***}$	23.8 ± 1.3	23.3 ± 2.3
IV	$3.0 \pm 1.1^{***}$	27.0 ± 1.9	22.3 ± 1.4
V	$5.8 \pm 1.3^{**}$	25.8 ± 2.3	21.8 ± 2.7
VIA	$5.8 \pm 0.8^{**}$	20.8 ± 1.3	23.8 ± 2.5
VIB	$5.5 \pm 1.4^{**}$	24.8 ± 2.5	21.3 ± 1.4
VII	$4.5 \pm 0.9^{**}$	23.0 ± 1.5	26.3 ± 3.2
VIII	$6.3 \pm 1.2^{***}$	24.3 ± 0.9	23.3 ± 1.8
IXA	$9.0 \pm 1.1^*$	20.3 ± 2.3	19.8 ± 1.6
IXB	$14.7 \pm 0.7^{***}$	23.5 ± 1.4	23.0 ± 0.9
X	$19.6 \pm 0.9^*$	23.9 ± 1.1	23.5 ± 0.9
Hemisphere			
Sim	$1.0 \pm 0.4^{***}$	21.0 ± 2.7	24.5 ± 1.3
Ans	$4.3 \pm 1.4^{***}$	23.8 ± 2.1	22.0 ± 1.8
Pm	$1.0 \pm 0.4^{***}$	24.8 ± 1.7	21.5 ± 1.0
Cop	$1.8 \pm 1.0^{***}$	20.8 ± 2.0	19.8 ± 1.2
Pfl	$17.9 \pm 1.1^{**}$	23.8 ± 0.7	23.5 ± 1.0
Fl	$20.0 \pm 0.6^*$	23.1 ± 0.9	26.1 ± 0.9

Cerebellar cortical Purkinje cells were counted in cresyl-violet-stained transverse sections. Values are cell numbers/folial length of $630 \mu\text{m}$ presented as means $\pm$ s.e.m. ($n = 10$). Comparisons were analysed by ANOVA followed by a post hoc Fisher's PLSD. ANOVAs for each cerebellar site were all significant. Post hoc comparisons of cell densities between $PrP^{-/-}$ and $PrP^{+/-}$ and between $PrP^{-/-}$ and $PrP^{+/+}$ are shown to be significant by asterisks (one asterisk $P < 0.01$; two $P < 0.001$; three $P < 0.0001$). Ans, ansiform lobule; Cop, copula pyramis; Fl, flocculus; Pfl, paraflocculus; Pm, paramedian lobule; Sim, simple lobule; I–X, vermal lobules.

putamen, thalamus and hypothalamus, revealed no significant abnormalities. Purkinje cells are known physiologically to express abundant PrP (ref. 9). Moreover, two major sources of afferents to the Purkinje neurons, the granule cells and inferior olive, remained morphologically intact. It is therefore most likely that loss of PrP in Purkinje cells is the primary cause of the death of these cells.

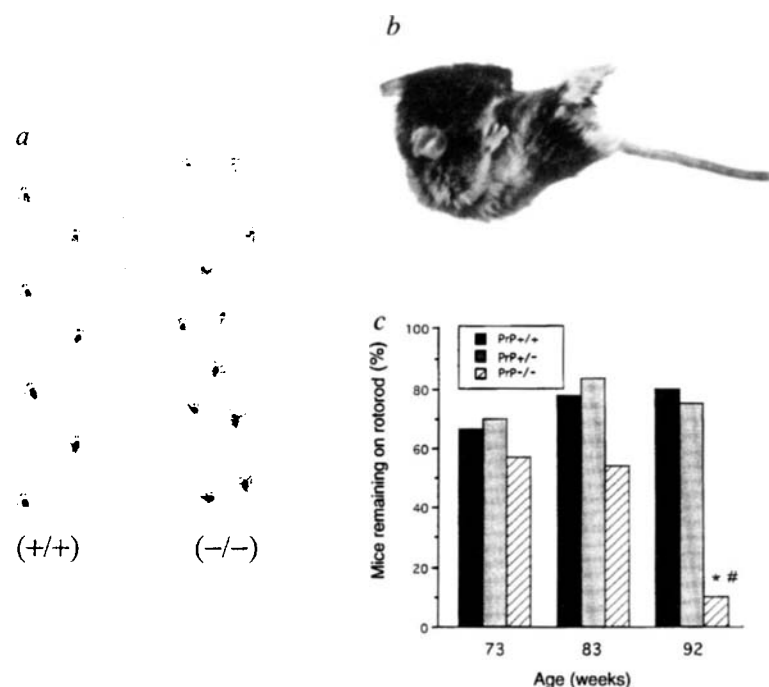


FIG. 1 Impaired motor coordination in aged mice homozygous for a disrupted PrP gene. *a*, Hind-footprint patterns of a $PrP^{-/-}$ mouse and a $PrP^{+/+}$ mouse aged 73 weeks. *b*, $PrP^{-/-}$ mouse aged 92 weeks that had fallen on walking and could no longer right itself. *c*, Rotarod test. Three age groups (73, 83 and 92 weeks old) of $PrP^{+/+}$ (black bars; $n = 14, 18$ and 5), $PrP^{+/-}$ (grey bars; $n = 63, 12$ and 4) and $PrP^{-/-}$ (hatched bars; $n = 14, 13$ and 10) mice were monitored on the rotarod. The performance of the oldest group of $PrP^{-/-}$ mice was significantly impaired compared with age-matched $PrP^{+/+}$ and $PrP^{+/-}$ mice (* $P < 0.05$) or the two younger groups of $PrP^{-/-}$ mice (# $P < 0.05$) (χ^2 test, Statview 4).

METHODS. PrP-null mice were generated as described². In the disrupted PrP loci, the 2.0-kb $XbaI$ – $EcoRI$ fragment containing the whole coding region was replaced with a neomycin-resistant gene, $PGK-neo$. Three series of F_2 offspring, including 39 $PrP^{-/-}$, 90 $PrP^{+/-}$ and 46 $PrP^{+/+}$ mice, derived from the same crosses between F_1 $PrP^{+/-}$ breeding pairs were fed under pathogen-free conditions in the same room at the Laboratory Animal Center for up to 95 weeks after birth. Footprints were obtained from mice with ink-painted hind paws induced to walk forwards on white paper. For the rotarod test, each mouse was placed on a rod (7 cm in diameter) rotating at 2 r.p.m., with its head against the direction of rotation. When the mouse managed to stay on the rotarod for over 180 seconds by the fifth trials, motor coordination was classed as normal. The roller was adjusted for the size of the mouse under investigation; 2 r.p.m. was the maximum speed of rotation at which all three 90-week-old wild-type mice performed normally in a preliminary experiment.

In contrast to our mice, two other lines of PrP-null mice^{7,8} had not produced the ataxic phenotype at up to 93 weeks. There could be a number of reasons for this difference. First, the genetic background of the mice may have led to a difference in phenotype. Although the mice of Büeler *et al.*⁷ have a similar genetic background to ours³, the different embryonic stem (ES) cell lines used by them (AB1; ref. 10), and by us (J1; ref. 11) are derived from distinct substrains of strain-129 mice. Subtle uncharacterized differences between the two might have affected the development of the phenotype. Alternatively, differences in breeding conditions may have contributed, and the phenotype may develop in the two other mouse lines at a later stage. To determine whether random integration of the targeting vector in our mice could have accounted for the observed phenotype, we used Southern blot analysis. Genomic DNA digested with several restriction enzymes was examined using three construct-derived DNA probes, including a *neo*-specific one, which between them encompassed about half of the construct sequence. The results confirmed the presence of a single copy of the *neo* gene and did not reveal any construct-derived sequence other than that in the *PrP* loci in the genome of our PrP-null mice (data not shown). The difference may also be due to a structural difference in mutated alleles. Büeler *et al.*⁷ replaced 552 base pairs of the PrP-coding region with a *neo* cassette, retaining the initial three codons and final 67 codons of the targeted mouse gene³. Manson *et al.*⁸ simply inserted a *neo* cassette into the PrP-coding region⁶. The new messenger RNA expressed by the disrupted *PrP* genes of these two lines might therefore allow retention of some aspects of normal PrP function. In contrast, we replaced the whole PrP-coding region with a *neo* cassette, excluding such a possibility². Results from the other two

lines of mice on their susceptibility to prions would appear to indicate that PrP is completely absent in these mice^{4,5,7,8}. But mutations in the other mouse lines may abrogate susceptibility to prion while allowing retention of some physiological functions of PrP. We cannot at present explain these differences in phenotype. Investigation of whether the phenotype can be rescued by a transgene encoding normal PrP (PrP^C) should confirm the relationship between the PrP-null mutation and its phenotype.

In the prion diseases, a protease-resistant isoform of PrP (PrP^{Sc}), thought to be generated post-translationally from PrP^C accumulates in affected brains. It has been proposed that there is a causal relationship between the presence of PrP^{Sc} and the pathological changes that occur in the brain.¹² Our findings suggest that the functional loss of PrP^C on the neuronal cell surface, caused by its continual conversion to PrP^{Sc}, is also involved in the pathogenesis of the prion diseases. At present we do not know the exact age at onset for ataxia and loss of Purkinje cells in *PrP*^{-/-} mice, but histology of the cerebellum is normal in null mice at 18 weeks, indicating that neuronal cell death occurs with ageing. The lateness of onset of cell loss suggests that PrP^C may play a role in the long-term survival of Purkinje cells. Electrophysiological studies of hippocampal slices of another line of *PrP*^{-/-} mice show that GABA_A-receptor-mediated fast inhibition is weakened¹³, an effect that was rescued by a transgene encoding human PrP^C (ref.

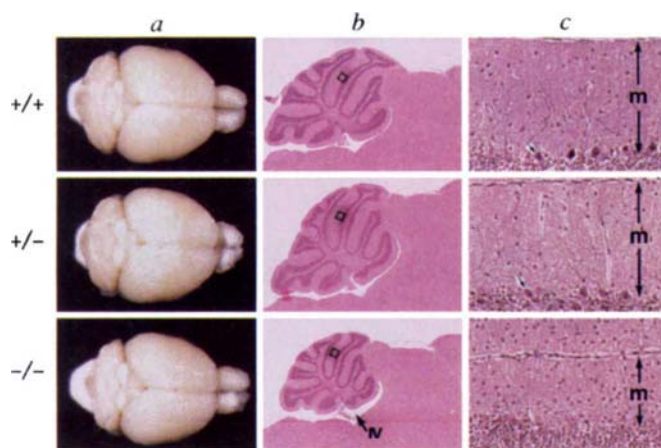


FIG. 2 Small cerebellum with an extensive loss of Purkinje cells in aged mice homozygous for a disrupted *PrP* gene. Macroscopic appearance of whole brains (a) and histology of parasagittal sections of the cerebella (b and c) are compared for *PrP*^{-/-}, *PrP*^{+/-} and *PrP*^{+/+} mice aged 83 weeks. Sections were stained with haematoxylin-eosin (original magnification in b, 10×) and also by Bodian's method (original magnification in c, 200×). c, Regions boxed in b; Purkinje cells are indicated by small arrows. The cerebellum of a *PrP*^{-/-} mouse is obviously smaller than those of *PrP*^{+/-} and *PrP*^{+/+} mice, so the fourth ventricle (IV) is dilated. The cerebellum section shows the extensive loss of Purkinje cells in a *PrP*^{-/-} mouse. The molecular layer (m) without Purkinje dendritic trees is thinner, resulting in greater density of nuclei of stellate neurons. Granule cells remain morphologically intact and there is no astrogliosis, as confirmed by immunostaining for glial fibrillary acidic protein (data not shown). All 12 *PrP*^{-/-} mice aged between 73 and 96 weeks examined so far show similar pathological changes in the cerebellum.

METHODS. Mice were perfused intracardially with a 4% paraformaldehyde solution buffered with 0.1 M phosphate buffer (pH 7.4) under pentobarbital anaesthesia. The brains were immediately removed and immersed in 4% paraformaldehyde. Paraffin-embedded cerebellar tissues were sagittally sectioned at 5-μm thickness. Sections were stained after deparaffinization.

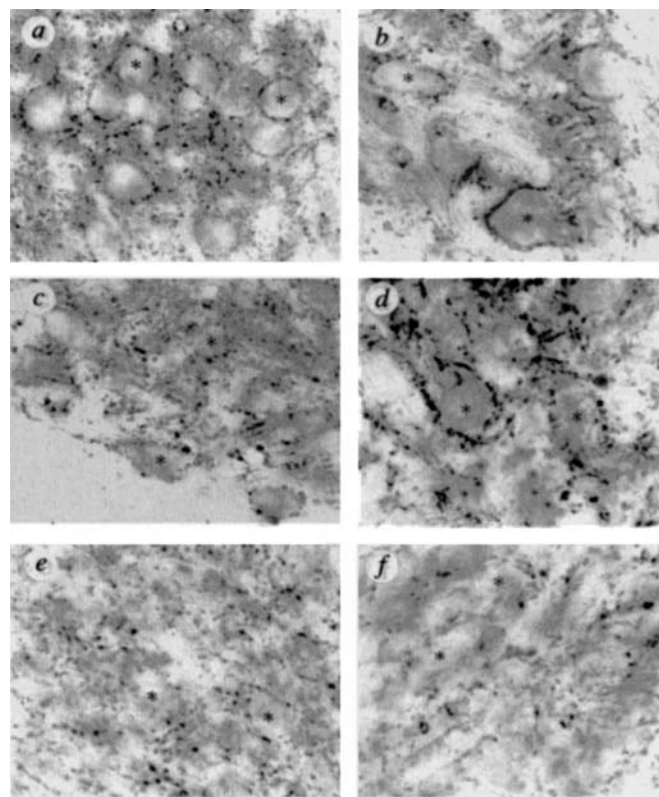


FIG. 3 Glutamate decarboxylase (GAD) immunostaining of cerebellar interpositus nucleus (original magnification in a, c and e, 560×) and lateral vestibular nucleus (b, d and f, 560×) taken from 96-week-old *PrP*^{+/+} (a, b), *PrP*^{+/-} (c, d) and *PrP*^{-/-} (e, f) mice. In *PrP*^{+/+} and *PrP*^{+/-} mice, many axons, bouton-like varicosities and chains of varicosities reveal the GAD immunolabel, whereas such labelled axonal/terminal structures are sparse and interspersed randomly in the neuropil in *PrP*^{-/-} mice. Asterisks indicate typical GAD-immunonegative neuronal perikarya seen with (a-d) or without (e, f) appreciable numbers of pericellular immunolabelled varicosities.

METHODS. Mice were perfused as described. Frozen coronal sections (40 μm thick) were immunostained using a polyclonal GAD antiserum (Chemicon, 1:2,500 dilution), avidin-biotin-peroxidase complex (Vector Labs) and diaminobenzidine.

531). This indicates that postsynaptic PrP^C is necessary for inhibitory synapses to be fully functional, and suggests that neuronal cell death in PrP^{-/-} mice may involve excitotoxic mechanisms¹⁵. A similar synaptic dysfunction is involved in the cerebellum and the resulting sustained overexcitation eventually causes death of Purkinje cells after a long incubation period. The loss of Purkinje cells in PrP-null mice probably mimics some aspects of neuronal cell death during ageing and in other chronic neurodegenerative conditions, so PrP-null mice will be valuable as a model for investigating neuronal cell death. □

Received 2 October 1995; accepted 31 January 1996.

1. Prusiner, S. B. *Rev. Microbiol.* **48**, 655–686 (1994).
2. Sakaguchi, S. *et al. J. Virol.* **69**, 7586–7592 (1995).
3. Büeler, H. *et al. Nature* **356**, 577–582 (1992).
4. Büeler, H. *et al. Cell* **73**, 1339–1347 (1993).
5. Prusiner, S. B. *et al. Proc. natn. Acad. Sci. U.S.A.* **90**, 10608–10612 (1993).
6. Manson, J. C. *et al. Molec. Neurobiol.* **8**, 121–127 (1994).
7. Büeler, H. *et al. Molec. Med.* **1**, 19–30 (1994).
8. Manson, J. C. *et al. Neurodegeneration* **3**, 331–340 (1994).
9. DeArmond, S. J. *et al. Neurology* **37**, 1271–1281 (1987).
10. McMahon, A. P. & Bradley, A. *Cell* **62**, 1073–1085 (1990).
11. Li, E., Bestor, T. H. & Jaenisch, R. *Cell* **69**, 915–926 (1992).
12. Forloni, G. *et al. Nature* **362**, 543–546 (1993).
13. Collinge, J. *et al. Nature* **370**, 295–297 (1994).
14. Whittington, M. A. *et al. Nature Genet.* **9**, 197–201 (1995).
15. Mattson, M. P. *Neuron* **4**, 105–117 (1990).

ACKNOWLEDGEMENTS. We thank K. Kurokawa, R. Nakaoke, I. Yoshimi, M. Nakano and H. Sato for technical assistance, and A. Nishida for help in manuscript preparation. This study has been supported in part by a grant from the Ministry of Health and Welfare of Japan.

CORRESPONDENCE and requests for materials should be addressed to S. K. (e-mail: katamine@net.nagasaki-u.ac.jp).

Simultaneous independent measurement of endocytosis and exocytosis

C. B. Smith & W. J. Betz

Department of Physiology and Neuroscience Program, University of Colorado Medical School, Denver, Colorado 80262, USA

STUDIES of membrane traffic between the cytoplasm and surface of a cell suggest that membrane internalization is tightly coupled to secretion. Studies using the capacitance technique show that endocytosis can follow evoked exocytosis within a second or less^{1–3}. The capacitance technique, however, measures only the net change in cell surface area, and thus separating exocytosis from endocytosis requires that the two events do not overlap in time. This condition is probably met with small, brief stimuli^{1–5}, but during prolonged stimulation it is more likely that exocytosis and endocytosis occur simultaneously⁶. We used FM1-43 fluorescence, which provides a cumulative measure of exocytosis, independent of endocytosis, in combination with capacitance monitoring to track unidirectional movements of membrane simultaneously and in real time in bovine adrenal chromaffin cells. We confirm that, with relatively small stimuli, exocytosis ceases before endocytosis begins (no overlap). In contrast, during prolonged stimulation, the onset of endocytosis is delayed by 2–3 min, but then the rate of endocytosis quickly grows to equal that of exocytosis. The delayed onset of endocytosis may be an emergency defence against catastrophic cell swelling.

Capacitance monitoring^{7,8} have proven valuable for measuring surface membrane area on a millisecond timescale in adrenal chromaffin^{3,7,9–11} and other^{12–16} cells. The fluorescent styryl dye FM1-43 has been useful for optical tracking of synaptic vesicle recycling in a variety of nerve terminals^{17–23}. FM1-43 reversibly stains, but does not penetrate, surface membranes of cells. Here we show that when bovine adrenal chromaffin cell secretory

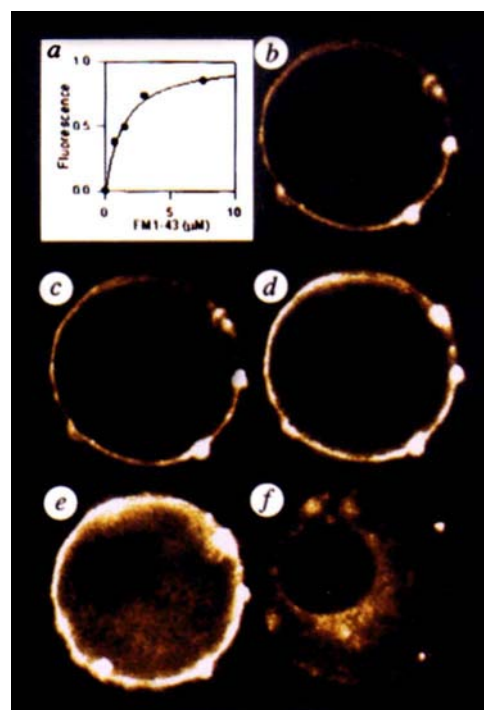


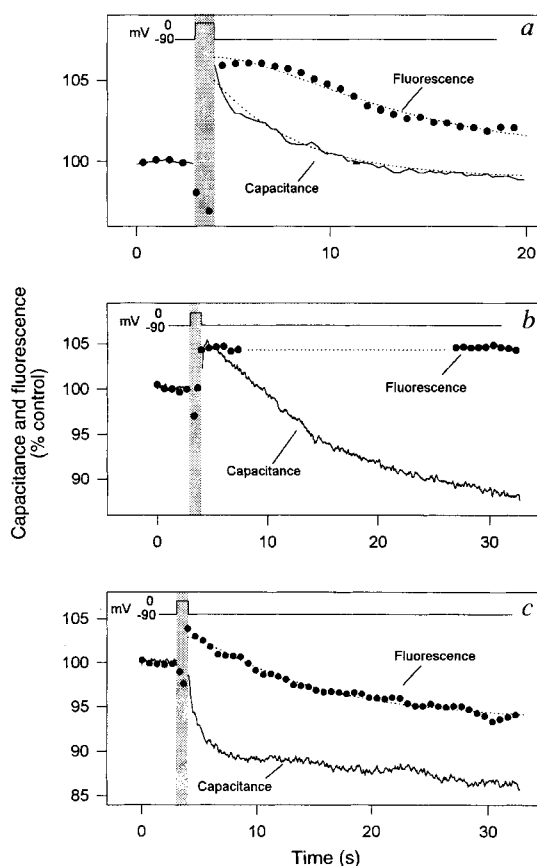
FIG. 1 Surface membrane staining and activity-dependent internalization of FM1-43. **a**, The average surface membrane fluorescence (y-axis) is plotted against FM1-43 concentration (x-axis). The solid line is drawn according to first-order reaction kinetics with $K_D = 1.33 \mu\text{M}$. **b**, Confocal image of a chromaffin cell in the presence of $4 \mu\text{M}$ FM1-43. Only the surface membrane is stained. Bright spots are adherent debris, which were excluded from analysis. **c**, Same cell, 5 min later. No noticeable change in fluorescence had occurred, indicating the absence of spontaneous exocytosis. **d**, Same cell, 15 s after addition of $10 \mu\text{M}$ nicotine to the Ringer-dye solution. The surface membrane fluorescence is brighter (by 32%), reflecting addition of surface membrane during exocytosis of secretory granules. **e**, Same cell, 5 min after addition of nicotine. Dye is now visible inside the cell. **f**, Same cell, 20 min after washing in nicotine-free, dye-free Ringer solution. Dye has been washed out of the surface membrane, and inside the cell the fluorescence is most intense around the nucleus.

METHODS. For isolation of the chromaffin cells the cortex was dissected from 2 large bovine adrenal glands acquired fresh from Monfort Biological (Greeley, CO). Each gland was perfused with 30 ml collagenase (0.5 mg ml^{-1} Type I; Worthington Enzymes, Freehold, NJ) 6–8 times at 10-min intervals in HEPES buffered DME/F-12 media (Gibco BRL, Gaithersburg, MD) and antibiotics (penicillin-G ($10^3 \text{ units ml}^{-1}$), streptomycin (1 mg ml^{-1}) and Fungizone ($25 \mu\text{g}$); Gemini Bioproducts Calabasas, CA) at 37°C . Medullary cells were gently triturated. The chromaffin cells were further purified in an isotonic (300 mOsm) 50% percoll solution (20-min centrifugation at 20,000 g), and directly plated in tissue culture dishes in DME/F-12/10% FBS (fetal bovine serum from Gemini Bioproducts, Calabasas, CA) at 5×10^5 cells per ml. The cells were incubated at 37°C in 95% O_2 /5% CO_2 and used 2–3 days after isolation. Before the experiment the cells were washed from the tissue culture dishes and replated on glass cover slips. These cells gave highly consistent and reproducible experimental results. After a gigohm seal was established (see methods, Fig. 2), individual cells usually could be held for more than 30 min during which time multiple responses could be obtained. Images in Fig. 1 were acquired with a Biorad MRC 600 laser scanning confocal imaging system mounted on a Nikon Optiphot microscope with Nikon $\times 63$ oil immersion objective, with 488 nm excitation filter, 510 nm emission filter, aperture setting 4, 1% transmittance neutral density filter, slow scan mode. The normal Ringer contained (in mM) 140 NaCl, 2.8 KCl, 10 HEPES, 2 MgCl_2 , 2.5 CaCl_2 , pH 7.2. FM1-43 (Molecular Probes, Eugene, OR) concentration was $4 \mu\text{M}$. All experiments were performed at 20 – 25°C .

granules undergo exocytosis with FM1-43 in the bathing solution, cell-surface fluorescence and membrane capacitance increase by equivalent amounts. Subsequent endocytosis causes capacitance to decrease, but fluorescence does not change (endosomes remain fluorescent). Thus we obtain independent and simultaneous

FIG. 2 Simultaneous monitoring of cell capacitance and edge fluorescence following electrical stimulation. *a*, Chromaffin cell membrane potential was clamped at -90 mV in the presence of $3 \mu\text{M}$ FM1-43 while cell capacitance and edge fluorescence were monitored simultaneously. After a 3-s control period (during which cell capacitance (6.2 pF) and fluorescence were constant) a depolarizing stimulus (to 0 mV for 1 s; shaded region) was given. During the depolarizing pulse, cell fluorescence decreased by about 3% , reflecting the voltage dependence of FM1-43 (see below). The trend in the fluorescence during depolarization varied because data points sometimes crossed the hatched border. After the stimulus, cell capacitance had increased by 5.9% and cell edge fluorescence had increased by 6.0% . The cell capacitance then returned to the prestimulus level with an exponential time course (dotted line; $\tau = 7.8$ s). The fluorescence remained elevated for several seconds, and then slowly decreased. The slow fluorescence decrease could be explained quantitatively (dotted line) by a model that assumed that endocytosed dye was photobleached at a constant rate ($\tau = 5.9$ s; see below). *b*, An experiment like that in *a*, except that the illumination was stopped during most of the post-stimulus period, which had the effect of preventing the slow decrease in the fluorescence signal, showing that the decrease was the result of photobleaching. Also, in this experiment the capacitance trace decreased below the control level, a phenomenon that was observed in about one-half of cells studied. *c*, Like *a*, but from an experiment exhibiting primed endocytosis ($\tau \approx 1.1$ s). FM1-43 fluorescence increased by 4% ; capacitance showed no increase, but decreased rapidly, falling to about 90% of prestimulus capacitance. Evidently the primed endocytosis masked exocytosis, which was detected by the fluorescence signal. The subsequent decrease in fluorescence could be explained by photobleaching (dotted line) as in *a*.

METHODS. Cell capacitance was monitored using the phase tracking method²⁹ based on a PC acquisition system running software written in PDS Basic 7.1 (Microsoft). The internal solution was of the following composition (in mM): 145 CsGlutamate, 10 HEPES 9.5 NaCl, 2 MgATP, 0.5 EGTA pH 7.2 . The external solution was as described in Fig. 1, with $3 \mu\text{M}$ FM1-43 added to the external solution. Both capacitance and fluorescence changes were blocked by omitting Ca^{2+} ions from the bathing solution (results not shown), suggesting that Na^+ channel charge mobilization did not contribute to the capacitance signals in bovine, as it does in rat adrenal chromaffin cells⁹. Cells were imaged at about 6 Hz with a silicon intensified target (SIT) camera (Dage 66) coupled to a Silicon Graphics workstation (Personal Iris 4D/35) running locally written software based on an image processing library (G. W. Hannaway). After the experiment, the average fluorescence of a $1\text{-}\mu\text{m}$ -wide rim of cell surface was measured for each image. The outer edge of the rim was marked manually by outlining the cell; regions with adherent debris and pipette contact were masked out. For plotting, $3\text{--}5$ sequential images ($0.5\text{--}0.8$ s) were averaged. The voltage dependence of FM1-43 was 3.4% per 100 mV potential change (dimming with depolarization). In theory, it should be possible to use this voltage dependence to monitor endocytosis optically, because sensitivity to surface membrane potential would disappear after endocytosis. The expected signal, however, is small (3.3% ($\Delta F/\Delta V_m$) of about 5% (fluorescence change with stimulation)) and we were not able reliably to detect fluorescence change associated with internalization with our techniques. The fits to the fluorescence curves (dotted lines) in *a* and *c* were calculated as follows. The decay of the capacitance curve was fitted by a single exponential (rate constant $k_1 = 0.128$ (*a*) and 0.88 (*c*) s^{-1}). This decay was assumed to reflect endocytosis, and it was assumed that endocytosed dye was photo-



bleached at a constant rate (k_2). (Presumably dye in the surface membrane was also photobleached, but was replaced by intact dye from the bathing medium.) This series of irreversible reactions is described simply as $A \rightarrow B \rightarrow C$, where A is the fluorescence increase due to externalized membrane (A_0 immediately after the stimulus, when $t = 0$), B is the fluorescence in endosomes, and C is the photobleached endosomal dye (is the A_0 when $t = \infty$). The total fluorescence signal F at any time is then given by the following:

$$F = F_0 + A_0 \left(e^{-k_1 t} + \frac{k_1 e^{-k_2 t}}{k_2 - k_1} (e^{(k_2 - k_1)t} - 1) \right)$$

where F_0 is the baseline (post-stimulus) fluorescence, and k_2 is the rate of photobleaching. After k_1 was determined from the capacitance curve, k_2 , A_0 and F_0 were determined by fitting the equation to the fluorescence data. The resulting values ($k_2 = 0.177$ (*a*) and 0.094 (*c*) s^{-1} ; $A_0 = 5.8\%$ (*a*) and 9.4% (*c*), and $F_0 = 100.6\%$ (*a*) and 93.5% (*c*)) produced reasonable fits to the fluorescence data (dotted lines).

measures of exocytosis (fluorescence) and endocytosis (capacitance minus fluorescence).

Figure 1 shows a dose-response curve (*a*, cell-surface average fluorescence versus FM1-43 concentration) and five confocal images of a chromaffin cell before (*b*, *c*) during (*d*, *e*) and after (*f*) stimulation with bath-applied nicotine ($10 \mu\text{M}$). FM1-43 ($4 \mu\text{M}$) was present in the bathing solution in all but the last image. Because the fluorescence of the dye is much higher in membranes than in water, it selectively stains the cell-surface membrane¹⁸. The images in Fig. 1*b* and *c*, acquired 5 min apart, are nearly identical, showing that there was no spontaneous exocytosis during the control period. The image in Fig. 1*d* was acquired 15 s after the addition of $10 \mu\text{M}$ nicotine; the surface brightened noticeably. Five min later (Fig. 1*e*), dye had spread into the interior of the cell (the nucleus remained unstained). Figure 1*f* was acquired 20 min after dye and nicotine were washed from the chamber; the cell surface was no longer fluorescent. In the

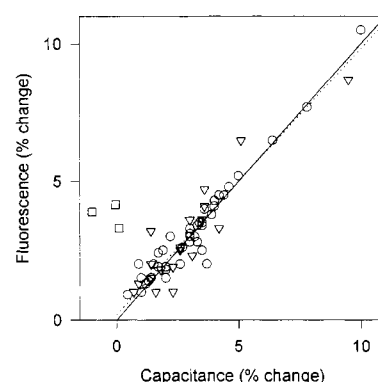
experiments described below, we concentrated only on the surface of the cell during and shortly after stimulation, that is, the transition from Fig. 1*c* to *d*.

Simultaneous capacitance and fluorescence measurements from three experiments are shown in Fig. 2. Fluorescence was measured as the average brightness of the cell surface ($1\text{-}\mu\text{m}$ -wide rim, with stained debris excluded from analysis). A 1 -s depolarization was applied (shaded region) after a 3 -s control period. Immediately after the depolarization, capacitance had increased by 5.9% (Fig. 2*a*). Surface fluorescence also had increased by a nearly identical amount (6.0%), presumably reflecting the staining of the membranes of the granules that had exocytosed during the stimulus. Note that during the depolarizing pulse the fluorescence decreased. This reflected the sensitivity of FM1-43 fluorescence (F) to changes in membrane potential (V_m ; $\Delta F/\Delta V_m = 3.4\%$ per 100 mV, dimming with depolarization^{24,25}).

The capacitance returned to baseline with a roughly exponen-

FIG. 3 Comparison of peak changes in cell capacitance (x-axis) and cell edge fluorescence (y-axis) for all experiments (61 events in 42 cells). In each case, the peak changes occurred immediately after the end of the depolarizing stimulus. Circles show responses to the first stimulus; triangles show responses to subsequent stimuli; squares show responses in cells giving primed endocytosis ($n = 3$). The solid line is the line of identity, and the dotted line is the linear regression (excluding squares). The two measurements agree well over the entire range, suggesting that FM1-43 fluorescence provides an accurate quantitative estimate of exocytosis.

METHODS. Capacitance and fluorescence values were determined as described above. In addition, we analysed the fluorescence images in more detail in an attempt to identify the location of individual exocytotic events. We examined regions (pixels) around the perimeter of the cell that brightened the most during stimulation. For example, if the cell capacitance increased by 5%, we identified the 5% of pixels that brightened the most, and then examined them for signs that might be expected to reflect exocytotic events at those locations. Results of all tests were negative, the main problem being noise from the SIT camera (despite the use of 5-frame averages). For example, a nearest neighbour analysis showed that the selected pixels were distributed no differently to those in controls (no stimulation). Nor did the selected pixels shift in radial position, from control to post-stimulation image, any differently to those in controls.



tial time course (dotted line). The fluorescence trace remained at its brightest level for several seconds, and then slowly declined. The slow decline was caused by photobleaching of internalized dye because the decline was entirely prevented simply by closing the shutter (Fig. 2b). The fluorescence decrease could be fitted with reasonable accuracy simply by assuming that endocytosed dye bleached at a constant rate (Fig. 2a, dotted line). Figure 2c shows a rare event (observed in 3 of 61 runs): primed endocytosis² completely obscured exocytosis on the capacitance trace, but not on the fluorescence trace.

Comparison of peak capacitance and fluorescence changes are shown for all experiments (61 runs from 42 cells) in Fig. 3. With three exceptions (squares), FM1-43 fluorescence matched faithfully the capacitance increase over the entire range of measurements. The simplest interpretation is that FM1-43 molecules, unlike hydrophobic ions²⁶, behaved the same whether dissolved in surface membrane or granule membrane, and thus provided a linear, quantitative measure of exocytosis. The same conclusion applies to stained endosomes because the fluorescence signal did not change after dye-labelled membrane was internalized (Fig. 2b).

The foregoing results (Figs 2 and 3) suggested that exo- and endocytosis usually occurred sequentially with brief depolarizing pulses. Another type of experiment that gave an altogether different result is illustrated in Fig. 4. In this case, a much stronger exocytotic stimulus was given by including $50 \mu\text{M Ca}^{2+}$ in the patch pipette⁶. The resulting capacitance increase was about 10 times larger than after a brief depolarizing pulse, and required much longer to develop. Capacitance (Fig. 4a, solid line) and fluorescence (circles) increased roughly linearly and by the same amount for the first 2–3 min, suggesting that only exocytosis was occurring during this time. Then the capacitance levelled off, a change that could have resulted from either cessation of exocytosis or onset of endocytosis. The continued rise in FM1-43 fluorescence suggests that exocytosis persisted unabated, which means that the capacitance trace levelled off owing to the delayed appearance of endocytosis, which quickly grew to equal the rate of exocytosis, producing the nearly flat capacitance trace.

What triggered the delayed initiation of endocytosis? The answer is not known with certainty, but we noted that the cell swelled for the first 2–3 min, and then stopped swelling (Fig. 4a, triangles), in close agreement with the capacitance trace. This suggests that cell swelling may have reached a threshold value (about 30% increase in surface area) that triggered massive endocytosis to limit further swelling.

In summary, the flat capacitance traces observed in cells both at

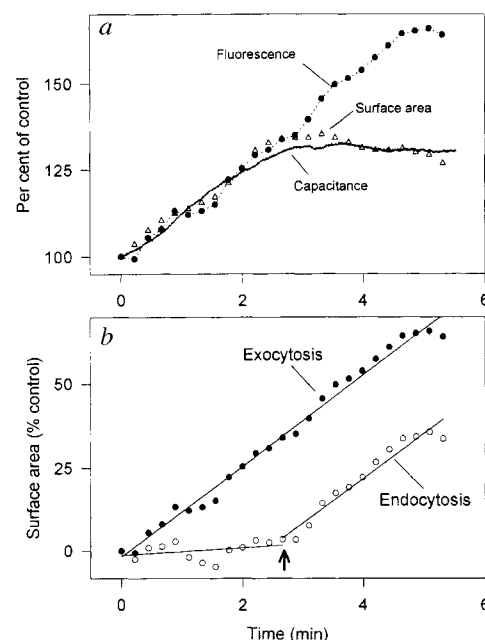


FIG. 4 a, FM1-43 fluorescence (circles), capacitance (solid line), and cell surface area (triangles) during intense and prolonged stimulation. The patch pipette contained Ca^{2+} buffered to $50 \mu\text{M}$ (refs 6,30), which evoked a massive increase in cell capacitance over a 2–3 min period (whole-cell configuration was established at zero time; the membrane potential was held at -90 mV throughout the experiment). During the first 3 min, capacitance and fluorescence increased at the same rate, suggesting that only exocytosis was occurring during this time. Thereafter, the capacitance trace levelled off, whereas the fluorescence continued to increase. The divergence suggests that endocytosis started abruptly after 3 min. The surface area of the cell, estimated from its diameter (assuming spherical geometry) also increased, and the swelling followed closely the capacitance change. b, Individual amounts of exocytosis (filled circles) and endocytosis (open circles) are plotted. Straight lines are linear regressions; two regressions are shown for endocytosis, before and after the onset of endocytosis (arrow). The slopes for exocytosis (13.6% per min) and endocytosis after the arrow (13.2% per min) are nearly identical.

METHODS. Measurements were performed as described in Fig. 2, except that fluorescence was measured from the entire cell, not just a $1 \mu\text{m}$ -wide surface rim. This was necessary because the cell was continuously swelling, making it virtually impossible to align successive images.

rest (Fig. 2) and after intense stimulation (Fig. 4) belie important differences in the physiological behaviour of the cells under the two conditions, differences that were revealed by simultaneous monitoring of FM1-43 fluorescence. □

Received 31 January; accepted 4 March 1996.

- Neher, E. & Zucker, R. *Neuron* **10**, 21–30 (1993).
- Thomas, P., Lee, A. K., Wong, J. G. & Almers, W. *J. Cell Biol.* **124**, 667–675 (1994).
- Artalejo, C. R., Henley, J. R., McNiven, M. A. & Palfrey, H. C. *Proc. natn. Acad. Sci. U.S.A.* **92**, 8328–8332 (1995).
- Artalejo, C. R., Elhamdani, A. & Palfrey, H. C. *Neuron* **16**, 195–205 (1996).
- von Gersdorff, H. & Møller, G. *Nature* **367**, 735–739 (1994).
- Augustine, G. J. & Neher, E. *J. Physiol.* **450**, 247–271 (1992).
- Neher, E. & Marty, A. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6712–6716 (1982).
- Joshi, C. & Fernandez, J. M. *Biophys. J.* **53**, 885–892 (1988).
- Horrigan, F. T. & Bookman, R. J. *Neuron* **13**, 1119–1129 (1994).
- Burgoyne, R. D. *Pflügers Arch.* **430**, 213–219 (1995).
- Mollard, P., Seward, E. P. & Nowicky, M. C. *Proc. natn. Acad. Sci. U.S.A.* **92**, 3065–3069 (1995).
- Heidelberg, R., Heinemann, C., Neher, E. & Matthews, G. *Nature* **371**, 513–515 (1994).
- Parsons, T. D., Lenz, D., Almers, W. & Roberts, W. M. *Neuron* **13**, 875–883 (1994).
- Rieke, F. & Schwartz, E. A. *Neuron* **13**, 863–873 (1994).
- Alvarez de Toledo, G., Fernandez-Chacon, R. & Fernandez, J. M. *Nature* **363**, 554–558 (1993).
- Hartmann, J., Scepek, S. & Lindau, M. *J. Physiol., Lond.* **483**, 201–209 (1995).
- Betz, W. J. & Bewick, G. S. *Science* **255**, 200–203 (1992).
- Betz, W. J., Mao, F. & Bewick, G. S. *J. Neuroscience* **12**, 363–375 (1992).
- Ryan, T. A. et al. *Neuron* **11**, 713–724 (1993).
- Betz, W. J. & Henkel, A. W. *J. Cell Biol.* **124**, 843–854 (1994).
- Kraszewski, K. et al. *Neuroscience* **15**, 4328–4342 (1995).
- Ryan, T. A. & Smith, S. J. *Neuron* **14**, 983–989 (1995).
- Henkel, A. W., Lübke, J. & Betz, W. J. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Grinvald, A., Hildesheim, R., Farber, I. C. & Anglister, L. *Biophys. J.* **39**, 301–308 (1982).
- Grinvald, A., Frostig, R. D., Lieke, E. & Hildesheim, R. *Physiol. Rev.* **68**, 1285–1366 (1988).
- Oberhauser, A. F. & Fernandez, J. M. *Biophys. J.* **69**, 451–459 (1995).
- Greer, M. A., Hagemann, F. C. & Illingsworth, D. R. *Hormone Metab. Res.* **24**, (1992).
- Hoffmann, E. K. & Dunham, P. B. *Int. Rev. Cytol.* **161**, 173–262 (1995).
- Fidler, N. & Fernandez, J. M. *Biophys. Journal* **56**, 1153–1162 (1989).
- Neher, E. *J. Physiol., Lond.* **395**, 193–214 (1988).

ACKNOWLEDGEMENTS. We thank M. Nowicky for assistance and encouragement setting up capacitance measurements, A. Fox, M. Lindau and D. O. Smith for help with software and S. Fadul, A. Henkel, L.-G. Wu and J. Angleson for their help.

CORRESPONDENCE and requests for materials should be addressed to W.J.B. (e-mail: bill.betz@uchsc.edu).

A candidate gene for the mouse mutation *tubby*

Konrad Noben-Trauth*, Juergen K. Naggert*, Michael A. North† & Patsy M. Nishina*

* The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, USA
† Sequana Therapeutic Inc., La Jolla, California 92037, USA

A mutation in the *tub* gene causes maturity-onset obesity, insulin resistance¹, and sensory deficits^{2,3}. In contrast to the rapid juvenile-onset weight gain seen in diabetes (*db*) and obese (*ob*) mice, obesity in *tubby* mice develops gradually, and strongly resembles the late-onset obesity seen in the human population. Excessive deposition of adipose tissue eventually leads to a twofold increase of body weight. *Tubby* mice also suffer retinal degeneration and neurosensory hearing loss^{2,3}. The tripartite character of the *tubby* phenotype shows striking similarity to human obesity syndromes, such as Alström⁴ and Bardet-Biedl⁵. Here we report the identification of a G → T transversion in a candidate gene that abolishes a donor splice site in the 3' coding region and results in a larger transcript containing the unspliced intron. This alteration is predicted to replace the 44-carboxy-terminal amino acids with a 20-amino-acid sequence not found in the wild-type protein. Additionally, a second, prematurely truncated transcript with the unspliced intron is observed in testis messenger RNA and a 2–3-fold increase in brain mRNA is observed in *tubby* mice compared to B6. The phenotypic features of *tubby* mice may be the result of cellular apoptosis triggered by expression of the mutated *tub* gene.

The autosomal recessive mutation *tubby* (*tub*), mapping to

chromosome 7, occurred spontaneously in a C57BL/6J (B6) mouse¹. In addition to slowly developing obesity, homozygous *tubby* mice gradually increase their plasma insulin levels and food intake, an apparent response to weight gain (our unpublished results). Despite hyperactivity of islet β-cells¹, *tubby* mice have almost normal glucose levels and, unlike many rodent models of obesity, are essentially normocorticaemic. *Tubby* mice develop a progressive retinal degeneration which is characterized by a loss of photoreceptor cells and results in an abnormal electroretinogram by 3 weeks of age². They also have some hearing loss² as a result of the degeneration of the organ of Corti and ganglion cells in the basal end of the cochlea³. These latter phenotypes have been named 'retinal degeneration-5' (*rd5*)². Because *rd5* was identified in the *tubby* stock, is absent from the parent B6 strain, and is tightly linked to *tub*^{2,6}, it seems likely that *tub* and *rd5* are the same gene. Because of the important multiple manifestations of the *tub* mutation, we investigated its molecular basis by positional cloning.

A high-resolution genetic map was produced from crosses segregating for the obesity and retinal-degeneration phenotypes. Both *tub* and *rd5* cosegregated in the 4,658 meioses tested, providing strong evidence that *tub* and *rd5* result from the same mutation. The maximum estimated genetic distance for the chromosome 7 segment containing *tub/rd5* is 0.13 ± 0.09 cM (6 recombinants/4,658 meioses; Fig. 1). A physical map of this area, estimated to be 600 kilobases (kb) long, was assembled using yeast artificial chromosomes (YAC) and P1 clones (Fig. 1). To identify candidate genes for *tub/rd5*, P1s and cosmids, made from YAC967d4, which spanned the 600 kb, were screened for coding sequences by exon trapping⁷ and complementary DNA from adult testis, brain and eyes of B6 mice was directly selected^{8,9}.

Selected cDNA clones (0.6–1.6 kb) and exon-trap products

FIG. 2 A G → T transversion in a donor splice site leads to aberrant transcripts in *tub/tub* mice. Northern analyses of brain and testis mRNA from B6 (+/+) and C57BL/6J-*tub/tub* mice are shown. a, Probe 2.61F-C13R, containing 1.6 kb of coding region, hybridizes to a 6.3-kb wild-type transcript and ~ 6.6-kb and 2.1-kb transcript in *tub* mice; b, Probe C13F3-C13R3, specific for the C-terminal intron, recognizes only *tub*-derived transcripts; and c, the 3'-specific probe c15 does not hybridize to the 2.1-kb transcript. RNA size markers (Gibco/BRL) are indicated in kilobases. d, C57BL/6J-*tub/tub* mice, but not congenic or B6, carry the G-to-T change at the first nucleotide of the C-terminal intron, resulting in a change in the donor splice site. Sequence surrounding the C-terminal intron from B6-*Hbb*⁹/*tubHbb*⁵, C57BL/6J and C57BL/6J-*tub/tub* is shown. B6-*Hbb*⁹/*tubHbb*⁵ is the congenic strain used to maintain the *tubby* stock. METHODS. Oligonucleotide primers used in this experiment are as follows: 2.61F, 5'-ACCTGAGGCAGCAGAAGCT-3'; C13R, 5'-CAGCCAGTCTGTGG-TTGGT-3'; C13F2, 5'-TGCAGAACAGACGCCAGT-3'; C13F3, 5'-GATGTTG-TACGCATGTGTC-3'; and C13R3, 5'-TGAGACAGGGAGACCAAGG-3'. For RNA preparation, whole organs from C57BL/6J and C57BL/6J-*tub/tub* mice were flash-frozen in liquid nitrogen, homogenized in 500 mM NaCl, 10 mM Tris, pH 7.2, 10 mM EDTA, 2% SDS, and incubated with 250 µg ml⁻¹ Proteinase K (EM Sciences) for 2 h at 37 °C. Oligo(dT) cellulose (Pharmacia) was shaken with the homogenate on an incubator for several hours then loaded onto a PolyPrep chromatography column (BioRad). After washing in 100 mM NaCl, 10 mM Tris, pH 7.2, 0.1 mM EDTA, poly(A)⁺ RNA was eluted in 10 mM Tris, pH 7.2, 10 mM EDTA. For northern blot analysis, 2–5 µg poly(A)⁺ RNA was fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond N⁺ membrane (Amersham) and hybridized with the indicated probes in the presence of 500 mM sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA at 65 °C. Blots were washed in 40 mM sodium phosphate, pH 7.2, 1% SDS, 1 mM EDTA at 65 °C, followed by a stringent wash in 0.1% SDS, 0.1 × SSC at 68 °C. Integrity, equal loading and transfer efficiency were assessed by control hybridization with a rat GAPDH probe. The intron-specific probe was generated by amplification of the genomic PCR product of C13F2 and C13R with oligonucleotide primers C13F3 and C13R3. Probe c15 was obtained by EcoRI digestion of the cDNA clone c15 from the cDNA selection. Probes were random labelled with [α -³²P]dCTP (Amersham). Genomic DNA PCR-amplified with oligonucleotide primers flanking the donor splice site, C13F2 and C13R, was gel-purified and manually sequenced by dideoxy cycle sequencing (Sequitum, Epicentre Technologies).

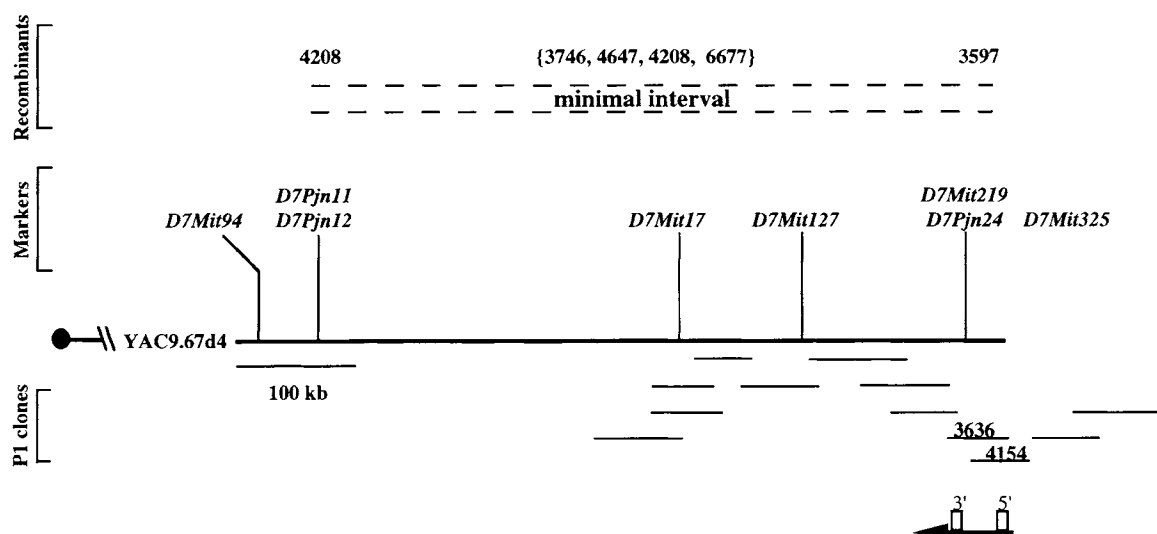
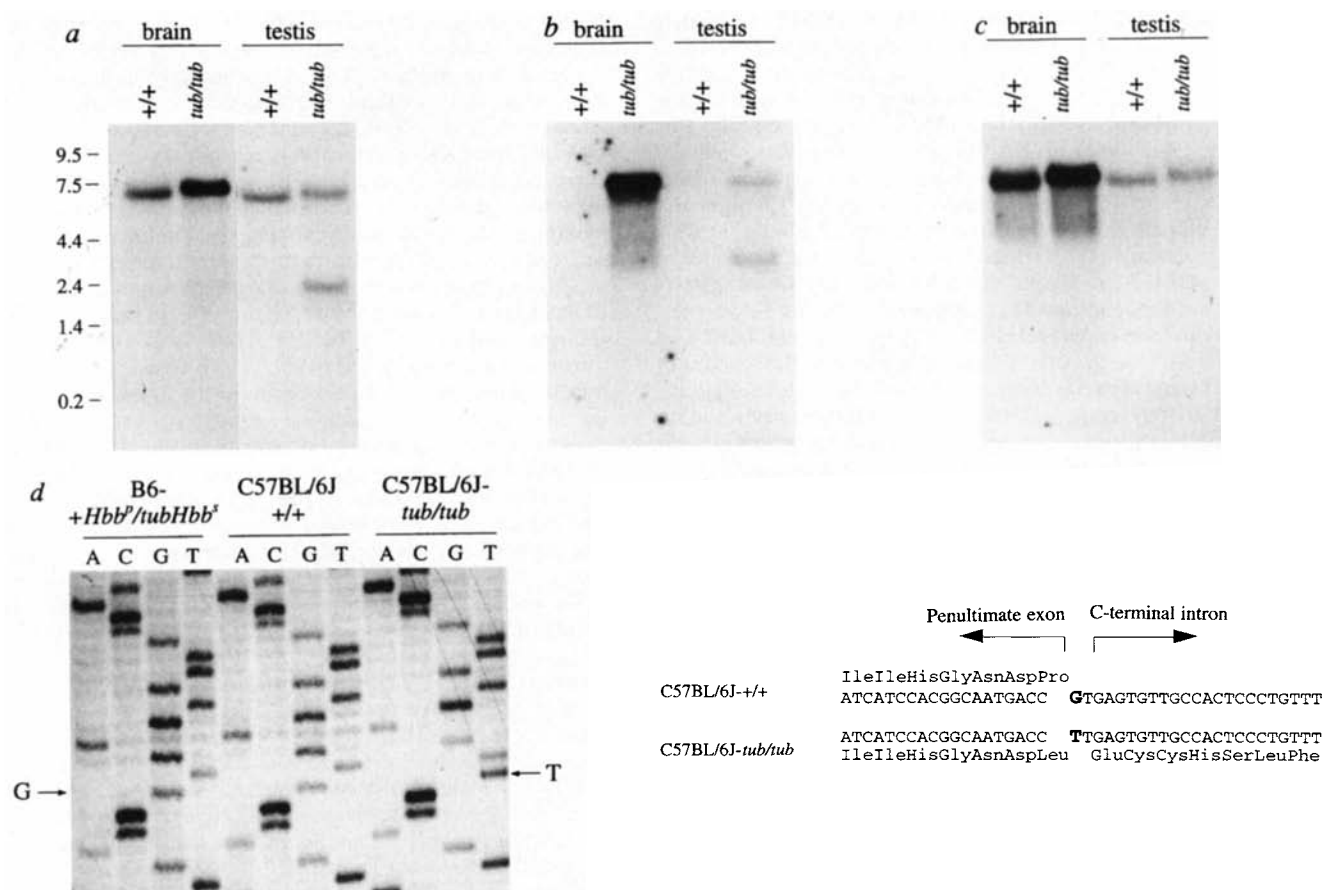


FIG. 1 Localization of the *tub/rd5* region on mouse chromosome 7. The genetic map is based upon 4,658 meioses from intercrosses or backcrosses of C57BL/6J-*tub/tub* mice with strains CAST/EiJ, NOD, NON-H2K^b, AKR/J or A/J mice. Recombinants that define the minimal interval are identified by pedigree number. Numbers in parentheses represent mice inheriting a recombinant chromosome in the minimal interval. These recombinant crossovers have not been assigned to a physical location in the contig. A physical contig of ~ 2 Mb was assembled using a combination of YAC, BAC and P1 clones (our unpublished results). The 5' end of the candidate gene maps to P1 3636 and 4154, whereas the 3' end maps only to P1 3636, indicating that the gene is transcribed from the telomere to the centromere.

METHODS. DNA was isolated from individual progeny and typed for simple sequence-length polymorphisms¹⁸. PCR amplification was done as described¹⁹. All recombinants were progeny-tested (minimum of 20 offspring) to confirm phenotypic classification. Primers were: *D7Pjn11* (F), 5'-TTCACAAAAGCACACCTGG-3'; (R), 5'-GTCCCAAGGATGGAGACCT-3'. *D7Pjn12* (F), 5'-TGGTGAGCAAAACAAGGAAC-3'; (R), 5'-TGGGGAAAGCA-ATTCTGG-3'. *D7Pjn24* (F), 5'-GCCTGTCAGCAAGGACCTT-3'; (R), 5'-CCAT-GTCCCAACAAGATGG-3'. YAC clones were obtained by PCR-screening of mouse YAC DNA pools (Research Genetics) and P1 clones were from Genome Systems.



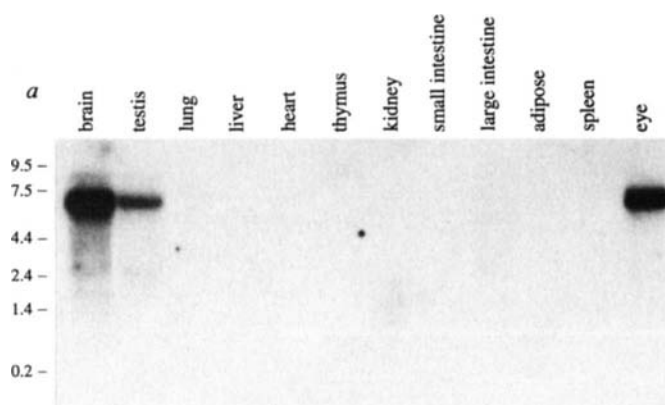


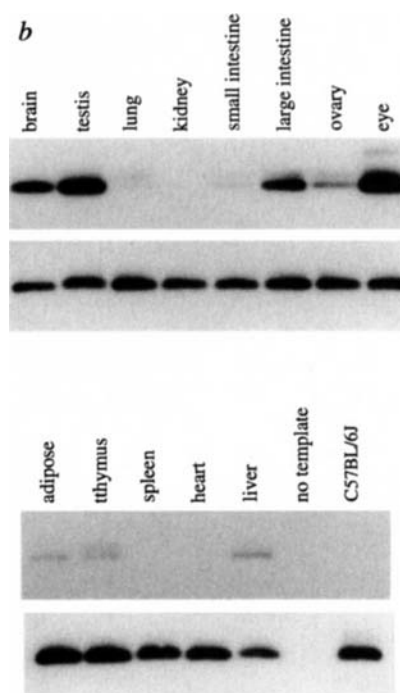
FIG. 3 Expression of the *tub* gene. **a**, Northern blot analysis of adult tissues shows that expression is strong in brain, eye and testis after 10 days exposure. **b**, RT-PCR was done with RNA from adult tissues and DNA from C57BL/6J using primers specific for nucleotides 1,080 to 1,423 (top band) or GAPDH (bottom, constant band). The *tub* gene-specific primers span 2 introns with a combined length of about 1 kb.

METHODS. Poly(A)⁺ RNA (2 µg) was treated with DNase I (Boehringer-Mannheim) and reverse-transcribed using the Superscript preamplification system (Gibco/BRL). PCR was performed using 5 ng single-stranded DNA, primer 2.40F (5'-GATGGCAAGAAGGTGTTCC-3'), 2.40R (5'-TCATTGCGGG-GGCGGATAC-3') and AmpliTaq (Perkin Elmer) under the cycling conditions: 95 °C for 1 min for denaturation, then 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s for 49 cycles, followed by 72 °C for 2 min. Forward and reverse GAPDH oligomers were 5'-ATGGTGAAGGTCGGTGTGA-3' and 5'-ACCACTA-GACTCCACGACAT-3', respectively. PCR products were electrophoresed in 1% agarose, transferred to Hybond N⁺ (Amersham) and hybridized with either 2.61F-C13R or GAPDH cDNA probes.

were sequenced and analysed for overlaps using the Assembly-LIGN program (Kodak). Sequences were evaluated for nucleic acid and protein similarities using search procedures¹⁰ such as BLASTN, BLASTP, tBLASTX and motif screening. Unique cDNA clones and single clones from groups of overlapping clones were hybridized to Southern blots of *Eco*RI-digested P1s. Positive clones mapping back to the minimal region were analysed by Southern blot analysis for genomic alterations and by northern analysis for aberrant expression between tubby and B6 mice.

One cDNA clone, c33, obtained from a DNA contig of overlapping sequences that included 12 cDNAs and 5 exon-trap products, showed an altered hybridization pattern to mRNA derived from tubby in that they expressed a slightly larger transcript in brain and testis (6.6 kb in tubby versus 6.3 kb in B6). In addition, there is an apparent 2–3-fold increase in the amount of the 6.6-kb brain transcript compared to the 6.3-kb product relative to a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) control. Furthermore, clone c33 identified a 2.1-kb transcript in testis mRNA derived from tubby mice which was not observed in B6 mice (Fig. 2).

To determine the molecular basis of these differences, oligonucleotide primers were made according to the cDNA sequences from the contig of overlapping clones. These primers were then used to amplify gene-specific fragments from cDNA and genomic DNA in the polymerase chain reaction (PCR). Several oligonucleotide combinations derived from the carboxy-terminal portion of the gene generated a PCR product from *tubby*-derived cDNA that was 400 base pairs (bp) longer than the product generated from B6-derived cDNA. When we compared the corresponding nucleotide sequence in this region between *tubby* and B6 genomic DNA, we found a G → T transversion in a donor splice site that changed the wild-type donor splice site consensus sequence from GTGAGT to TTGAGT (Fig. 4). To confirm that the larger transcript in *tub/tub* mice was due to the presence of this unspliced carboxy-terminal intron, a PCR-generated probe specific for the



intron was hybridized to a northern blot of mRNA from tubby and B6 mice. As can be seen from Fig. 2, the probe detected a transcript in the mRNA from tubby mice but not from wild type.

Comparison of the sequences surrounding this donor splice site in standard inbred strains from historically independent lineages (AKR/J, BALB/cJ, DBA/2J and two wild-derived strains CZE-CHII/Ei and SKIVE/Ei), as well as from rat and rabbit, showed complete conservation of the B6 sequence, suggesting that the nucleotide change is not a normal allelic variation but a mutation leading to abnormal transcript (Fig. 2). The second 2.1-kb transcript in testis mRNA, which is only seen in tubby mice, probably arises from truncation of the full-length transcript as a result of the utilization of another polyadenylation site, perhaps contained in the unspliced intron. Probe c15, which lies 3' of the unspliced intron, does not hybridize to the 2.1-kb transcript, whereas the intron-specific probe hybridizes only to the transcripts from tubby mice.

Expression of the *tub* candidate gene, assessed by northern blot analysis, is predominant in brain, eye and testis (Fig. 3). Using the more sensitive reverse transcription PCR assay (RT-PCR), we also detected moderate gene expression in the large intestine and low expression in adipose, small intestine, lung, ovary, thymus and liver of adult mice (Fig. 3).

To assemble full-length cDNA sequences of the *tub* gene in brain and testis, we screened a mouse testis cDNA library (Stratagene) and rapidly amplified the 5' and 3' ends on brain cDNA (Clontech). The 6.3-kb mRNA contains a 1,584-bp open reading frame that could encode, beginning at the first ATG codon, a novel 505-amino-acid protein (Fig. 4). Preliminary sequence analysis of genomic and cDNAs from brain and testis indicates that alternative splice variants are present which change the amino-terminal sequence, possibly in a tissue-specific manner (results not shown). The predominantly hydrophilic nature of the predicted amino-acid sequence of the brain form suggests a cytosolic localization for this protein. The 200 carboxy-terminal

FIG. 4 a, Predicted amino-acid sequence of the brain transcript for the candidate gene mutated in the *tub* mouse. The first ATG in the open reading frame was assumed to be the translational start codon. b, Comparison of the C-terminal sequences of the *tub*/*rd5* gene product from wild-type and tubby mice. The mutation in the splice donor site is indicated by a bold letter. A potential cryptic polyadenylation signal in the tubby transcript is underlined. c, Alignment of the conserved region among Tub, CEEAL48.2K and mouse P46 proteins. Identity indicated by upper-case letters.

METHODS. A mouse testis oligo(dT)-primed cDNA library (Stratagene) was screened according to the manufacturer's instructions with the 1.6-kb 2.61F-C13R PCR probe, identifying 24 plaques, two of which were purified and sequenced automatically (Prism, Applied Biosystems). To isolate the flanking 5' and 3' sequences, the Marathon-Ready cDNA kit (Clontech) was used according to the manufacturer's protocol. The protein alignment was obtained using the MACAW program²⁰.

amino acids show a strong similarity (62% identity) to a putative mouse testis-specific phosphodiesterase, p4-6 (X69827)¹¹, as well as to the *Caenorhabditis elegans* 48.2K protein (Q09306, 59% identity)¹² Fig. 4. The amino-terminal portion of the candidate protein shows no strong similarity to known proteins (BLASTP)¹⁰. The biochemical function of the *C. elegans* 48.2K protein is not known. The mouse *p4-6* gene was identified by hybridization of a rat brain phosphodiesterase (PDE) probe to a mouse testis cDNA library and has 20–40% similarity to PDE, but it has not been characterized beyond its sequence. Although this new family of genes may function as phosphodiesterases, all three members lack the zinc-binding-site motifs characteristic of many, but not all, phosphodiesterases^{13,14}.

A precedent already exists for a molecular defect in a phosphodiesterase that leads to retinal degeneration¹⁵. Mutations in the β -subunit of cyclic GMP phosphodiesterase cause retinal degeneration in mice with the *rd1* mutation¹⁵ and in humans¹⁶. In *rd1/rd1* mice, an abnormal accumulation of cGMP appears to trigger apoptosis of the photoreceptor cells¹⁷. Hallmarks of apoptosis, such as pyknotic nuclei in photoreceptor cells in the absence of an inflammatory response, and an even distribution of cell loss in the photoreceptor cell layer, distinct from necrosis, have been observed in tubby mice³. Cochlear degeneration, maturity-onset obesity and insulin resistance may all result from similar tissue-specific apoptotic events that are triggered by

a

MTSKPHSDWI	PYSVLDDDEGS	NLRQQLDRQ	RALLEQKQKK	KRQEPLMVQA	NADGRPRSR	60
ARQSEEQAPL	VESYLSSSGS	TSYQVQEADS	IASVQLGATR	PPAPASAKKS	KGAAASGGQG	120
GAPRKEKKGK	HKGTSGPATL	AEDKSEAQGP	VQILTVGQSD	HDKDAGETAA	GGGAQPSGQD	180
LRATMQRKGI	SSSMFDEDE	DEDENSSSSS	QLNSNTRPSS	ATSRKSIREA	ASAPSPAAP	240
PPVDIEVQDL	EEFALRPAPQ	GITIKCRITR	DKKGMDRGMY	PTYFLHLDR	DGKKVFLLAG	300
RKRKSKTSN	YLISVDPTDL	SRGGDSYIGK	LRSNLMGTKF	TVYDNGVNPQ	KASSSTLES	360
TLRQELAAVC	YETNVLGFKG	PRKMSVIVPG	MNMVHERVCI	RPRNEHETLL	ARWQNKNTES	420
IIELOKNTFV	WNDDTQSYVL	NFHGRVTQAS	VKNFQIIHGN	DPDYIVMQFG	RVAEDVFTMD	480
YNYPLCALQA	FAIALSSFDS	KLACE				505

b

>intron_~0.4kb
|

	1570	1580	1590						
	AsnPhe	GlnIleIle	HisGlyAsnAsp	Pro					
C57BL/6J	ACTT	CCAGATCATC	CACGCAATG	ACC-----					
C57BL/6J- <i>tub/tub</i>	ACTT	CCAGATCATC	CACGCAATG	ACCTTGAGTG	TIGCCACTCC	CTGTTTITGA	TGTGTG		
	AsnPhe	GlnIleIle	HisGlyAsnAsp	LeuGluCys	CysHisSer	LeuPheLeuMet	Leu>		
		1580	1590	1600	1610	1620			
C57BL/6J	-----	-----	-----	-----	-----	-----	-----		
C57BL/6J- <i>tub/tub</i>	ACGC	ATGGTGCCCA	GCCCCACCC	CACCCCAAT	CCCCTGATCT	GGTCCATATC	AGCCAG		
	TyrAla	TrpCysPro	AlaProThrPro	ProProIle	Pro***				
	1630	1640	1650	1660	1670	1690			
C57BL/6J	-----	-----	-----	-----	-----	-----	-----		
C57BL/6J- <i>tub/tub</i>	TGAT	GGGATGTGGG	TATATGGCTT	TGTGTAGAAC	TTTCTAACTG	TAGTGATCTA	GAGTCC		
	1700	1710	1720	1730	1740	1750			
C57BL/6J	-----	-----	-----	-----	-----	-----	-----		
C57BL/6J- <i>tub/tub</i>	TGCC	CCTAGTGTCC	TGCATGTCTG	GGGCTTGGGA	ATACCCITTA	AATGGATGTC	TTTCTC		
	1760	1770	1780	1790	1800	1810			
C57BL/6J	-----	-----	-----	-----	-----	-----	-----		
C57BL/6J- <i>tub/tub</i>	CTCC	TGGGCCCTGC	TGCTGTGTG	CATCTCCCC	CTTCACCTC	TTCCTTCATA	ATGTTT		
	1820	1830	1840	1850	1860	1870			
C57BL/6J	-----	-----	-----	-----	-----	-----	-----		
C57BL/6J- <i>tub/tub</i>	CTCT	TGAACCTTTG	TTTGTTCAT	CCTTTCGATC	TCCTTGGCAT	TTCTGCTTTC	TCCTTC		
	1880	1890	1900	1910	1920	1930			
C57BL/6J	-----	-----	-----	-----	-----	-----	-----		
C57BL/6J- <i>tub/tub</i>	CCTC	TGTGGGCCA	TGTCTTACCT	GGTCTCCCTG	TCTCCACCAA	TTCTTGTCTG	GTGCAT		
	1940	1950	1960	1970	1980	1990			
C57BL/6J		1600	1610	1620	1630	1640			
		AspTyr	IleValMet	GlnPheGlyArg	ValAlaGlu	AspValPhe	ThrMet		
C57BL/6J	----	CGGACTA	CATCGTCATG	CAGTTTGCC	GGGTAGCAGA	AGATGTGTTC	ACCATG		
C57BL/6J- <i>tub/tub</i>	GCCA	CAGCGACTA	CATCGTCATG	CAGTTTGCC	GGGTAGCAGA	AGATGTGTTC	ACCATG		
	2000	2010	2020	2030	2040	2050			

c

CAEEL48.2K	knnLakFVedPavehclyKCsITRqKsGvDKGMFPTYFLHLefDtdKrqkiFLLaAaRR
TUB	vqDLeeFaLrPAPqgitiKCRITRdKkGMDrGMyPTyFLHLdrEDG-Kkv--FLLAGRKR
mouse P46	geDmEayVLvPAPrdhmvqwrIvRnKhGMDKGMFPsYyLyLEgEDG---VahFLLAGRKR
CAEEL48.2K	KKStTaNYLIStDPTnLSReGegYcaKVRSNaLGTqTfVVDsGqNpKt---tnhAaIR
TUB	KKSKTSNYLISvDPTDLsRGGSyIGKLRsNlmgTKFTVYDNGVNPqKaSSstleSgtlR
mouse P46	KrSKTSNYLISIDpKdmsRnGsnfvGKVRsNLGTkFTifDNGVNPPer-SywwpdsArIR
CAEEL48.2K	QELAAVcYETNVLGFKGPRKMTivmPgIepptEnrpavRcpVRPiqDkhTLLeRyrlndl
TUB	QELAAVCYETNVLGFKGPRKMSVivPGMnmvhe-----RvcirRrNeheTLLeRwQnknt
mouse P46	eELgvVCYETNVLGFRGPRKMTVilPGMdsrkq-----RmkVqPqNDqdsilsRvQkgag
CAEEL48.2K	dSLkiLSnksPqWNETQSYVLNFHGRVTQASVKNFQIIHqssPeYIVMQFGRisdDeFT
TUB	eSiieLQNKtPvWNETQSYVLNFHGRVTQASVKNFQIIHqndPDYIVMQFGRVAdvFT
mouse P46	hgLlLlQNKaPsWsDEsgayVLNFHGRVsrASVKNFQIVHpdPdhlVLQFGRVApniFT
CAEEL48.2K	MDFRYPLsAvQAFaIamtSFhGKLAynpgkswwqerqkcpgifq
TUB	MDynYPLCALQAFaIALSSFDSKLACE-----
mouse P46	MDFRYPLcLQAFaIcLSSFdGKLACE-----

the expression of the mutated candidate gene described here. Determining the cellular localization and function of the *tub* gene may identify new pathways relevant to the aetiology of common maturity-onset obesity and retinal degeneration. □

Received 11 March; accepted 27 March 1996.

1. Coleman, D. L. & Eicher, E. M. *J. Heredity* **81**, 424–427 (1990).
2. Heckenlively, J. R. et al. *Proc. natn. Acad. Sci. U.S.A.* **92**, 11100–11104 (1995).
3. Ohlemiller, K. K. et al. *NeuroReport* **6**, 845–849 (1995).
4. Alström, C. H., Hallgren, B., Nilsson, L. B., & Asander, H. *Acta psychiat. neurol. scand.* **129**, 1–35 (1959).
5. Bray, G. A. *Obesity Res.* **3**, 383–403 (1995).
6. Jones, J. M., Meisler, M. H., Seldin, M. F., Lee, B. K. & Eicher, E. M. *Genomics* **14**, 197–199 (1992).
7. North, M. et al. *Mamm. Gen.* **4**, 466–474 (1993).
8. Lovett, M. *Current Protocols in Human Genetics* (eds Dracopoli, N. C. et al.) 6.3.1–13 (Current Protocols, New York, 1994).
9. Segre, J. A., Nemhauser, J. L., Taylor, B. A., Nadeau, J. H. & Lander, E. S. *Genomics* **28**, 549–559 (1995).
10. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. *J. molec. Biol.* **215**, 403–410 (1990).
11. Varnubas, V. & Wolgemuth, D. J. *Biochim. biophys. Acta* **1217**, 203–206 (1994).
12. Wilson, R. et al. *Nature* **368**, 32–38 (1994).
13. Beavo, J. A. *Physiol. Rev.* **75**, 725–748 (1995).
14. Lacombe, M.-L., Podgorski, G. J., Franke, J. & Kessin, R. H. *J. biol. Chem.* **261**, 16811–16817 (1986).
15. Bowes, C. et al. *Nature* **347**, 677–680 (1990).
16. Farber, D. B., Flannery, J. G. & Bowes-Rickman, C. *Prog. Ret. Eye Res.* **13**, 31–64 (1994).
17. Wong, P. *Biochem. Cell Biol.* **72**, 489–498 (1994).
18. Dietrich, W. F. et al. *Nature Genet.* **7**, 220–245 (1994).
19. Naggert, J. K. et al. *Nature Genet.* **10**, 135–141 (1995).
20. Schuler, G. D., Altschul, S. F. & Lipman, D. J. *Proteins Struct. Funct. Genet.* **9**, 180–190 (1991).

ACKNOWLEDGEMENTS. We thank C. Avery, G. York and D. Young for mouse colony management; G. Bouchard-Collins, D. Burkart, N. Hawes, T. Maddatu, J. Hutchinson, E. Furlong and D. McMinim for technical assistance; E. Snyder and Y. Pouliot for bioinformatics support; and E. Eicher, V. Letts, W. Frankel, K. Paigen, B. Knowles, J. Todd, N. Dracopoli and T. Harris for discussions and advice. This work was supported by a grant from Sequana Therapeutics, Inc. Institutional shared services were supported by a National Cancer Institute Cancer Center support grant. K.N.T. is a recipient of a DFG fellowship.

CORRESPONDENCE and requests for materials should be addressed to P.M.N. (e-mail: prmn@aretha.jax.org). Sequences for the candidate *tub* gene have been deposited in Genbank, accession numbers U52433 (brain) and U52824 (testis).

A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125^{FAK}

Alan Richardson & J. Thomas Parsons

Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, Virginia 22908, USA

FOCAL adhesion kinase^{1,2} (pp125^{FAK}) is a member of a growing family of structurally distinct protein tyrosine kinases that includes the recently identified FakB³ and PYK2/CAKβ/RAFTK^{4,6}. Activation of pp125^{FAK} has been functionally linked to the formation of focal adhesions, integrin-mediated sites of contact between the cell and the extracellular matrix^{7,8}. The carboxy-terminal domain of pp125^{FAK} is also expressed as a separate protein called pp41/43^{FRNK} (where FRNK represents pp125^{FAK}-related non-kinase)⁹. Here we show that pp41/43^{FRNK} acts as an inhibitor of pp125^{FAK} by transiently blocking the formation of focal adhesions on fibronectin and constitutively reducing tyrosine phosphorylation of both pp125^{FAK} and of two focal adhesion proteins, tensin and paxillin. These inhibitory effects of pp41/43^{FRNK} are reversed by co-expression of pp125^{FAK}, suggesting that pp125^{FAK} and pp41/43^{FRNK} compete for a common binding protein(s) whose association with pp125^{FAK} is necessary for signalling by pp125^{FAK}. We propose that pp41/43^{FRNK} functions as an endogenous regulator of pp125^{FAK}, thus providing an unusual means to regulate both tyrosine kinase activity and cellular adhesion to the extracellular matrix.

To investigate effects of pp41/43^{FRNK} overexpression, chicken embryo (CE) cells were collected by trypsinization and allowed to

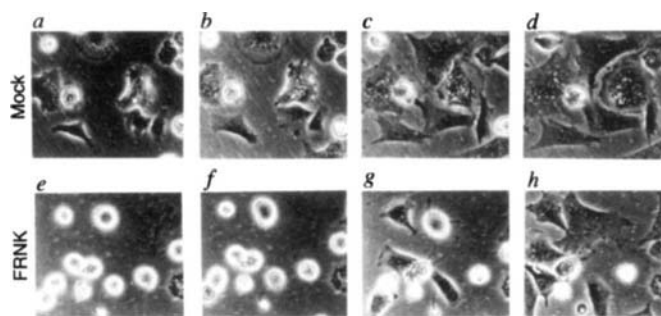


FIG. 1 Inhibition of CE cell spreading on fibronectin by pp41/43^{FRNK}. CE cells infected with RCAS (a replication-competent avian retrovirus) lacking an insert ('MOCK'; a–d) or containing sequences encoding FRNK (e–h) were allowed to spread on fibronectin-coated coverslips and photographed after 12 min (a, e), 20 min (b, f), 60 min (c, g) or 120 min (d, h). The number of cells spread on fibronectin after 20 min were counted (i). Bars: 1, mock-infected cells; 2, RCAS–FAK-infected cells; 3, RCAS–FRNK-infected cells; 4, cells infected with both RCAS–FAK and RCAS–FRNK.

METHODS. CE cells were transfected with RCAS DNA containing the coding sequences for pp41/43^{FRNK} (RCAS–FRNK) or pp125^{FAK} (RCAS–FAK) and cultured for 7–10 d. To co-express pp41/43^{FRNK} and pp125^{FAK} in the same CE cells, FAK and FRNK coding sequences were inserted into RCAS vectors encoding envelope proteins of either subgroup A or B. One week after transfecting CE cells with either RCAS(A)–FRNK or RCAS(B)–FAK, the cells were mixed and grown for a further week to allow dual infection of the cells with both virus subgroups. In some experiments cells were infected first with either RCAS–FRNK or RCAS–FAK and subsequently co-cultured with cells infected with RCAS A or B lacking an insert. To verify that pp41/43^{FRNK} and pp125^{FAK} were co-expressed in the same cell, an epitope-tagged variant of pp41/43^{FRNK} was co-expressed with pp125^{FAK} as described. Immunostaining of cells with a mAb to the epitope revealed expression of tagged pp41/43^{FRNK} in 86 ± 4% (n = 5) of the cells, confirming the dual infection of most of the cells in the population. For spreading experiments, cells were collected by trypsinization and washed twice with 1 mg ml⁻¹ soybean trypsin inhibitor in PBS. 10⁶ cells were added to 60-mm fibronectin-coated (1 µg/cm²) plastic dishes containing 2 ml of L15 medium (GIBCO). Cells were allowed to spread for the indicated times, photographed, coded and the extent of cell spreading assessed by 5 individuals. Unspread cells were defined as round phase-bright cells; spread cells were defined as cells with extended processes, lacking a rounded morphology and not phase-bright. Fewer than 5% of the cells could not be categorized as either of these types.

attach and spread on fibronectin-coated dishes. We observed that CE cells overexpressing pp41/43^{FRNK} spread more slowly on fibronectin than their counterparts transfected with empty vector ('mock cells'). Mock cells began to spread within 10 min of plating (Fig. 1a) and most of them attained a flat morphology between 20 and 60 min (Fig. 1b–d). In contrast, cells expressing pp41/43^{FRNK} were still round at 20 min (Fig. 1e, f); by 60 min only a portion of the cells had spread (Fig. 1g). The majority of cells expressing pp41/43^{FRNK} had spread by 120 min (Fig. 1h). This delay in spreading was quantified by counting the number of cells with a flattened appearance 20 min after plating on fibronectin. Significantly more ($P < 1 \times 10^{-5}$) mock cells had spread compared with cells expressing pp41/43^{FRNK} (Fig. 1i). The delay in cell spreading was reversed by co-expression of pp125^{FAK} with pp41/43^{FRNK}: after 20 min, significantly more ($P < 0.005$) cells expressing pp125^{FAK} and pp41/43^{FRNK} had spread than cells expressing pp41/43^{FRNK} alone.

Before formation of focal adhesions, structures termed 'focal complexes' are formed¹⁰. Such structures appear to represent immature focal adhesions, and can be detected as regions of intense vinculin staining around the cell periphery. Expression of pp41/43^{FRNK} delayed the appearance of focal complexes (Fig. 2a, b, d, e), suggesting that the delay in cell spreading was due to an early inhibition of recruitment of focal adhesion proteins into complexes. Similar results were also obtained when cells were immunostained with an antibody directed against pp125^{FAK} (data

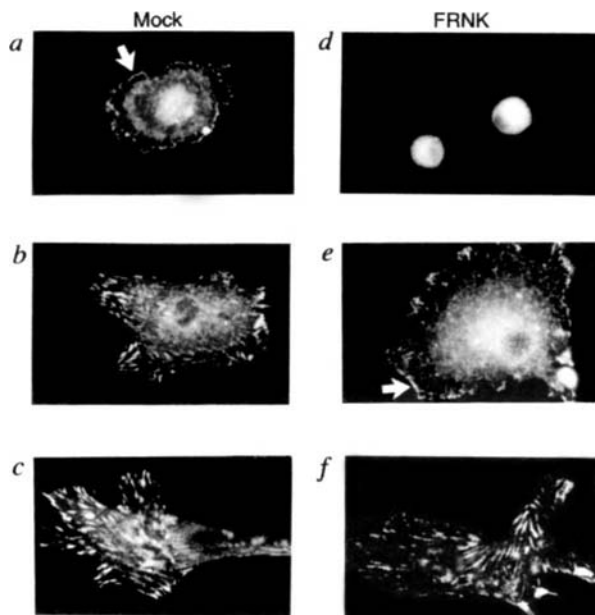


FIG. 2 Expression of pp41/43^{FRNK} delays focal complex formation. CE cells infected with RCAS lacking an insert ('MOCK'; a–c) or with RCAS–FRNK (d–f) were allowed to spread on fibronectin-coated coverslips for either 20 min (a, d), 60 min (b, e) or 15 h (c, f), then immunostained with an antibody against vinculin. Arrows indicate examples of focal complexes.

METHODS. CE cells were allowed to spread on fibronectin as described for Fig. 1. Cells were fixed with 3% paraformaldehyde after the indicated time, permeabilized with 0.4% Triton X-100 and stained with anti-vinculin (1/1,000 mAb VIN-11-5; Sigma) and subsequently with FITC-rabbit anti-mouse IgG (5 µg ml⁻¹).

not shown). Cells that were allowed to adhere to fibronectin for up to 15 h and stained with anti-vinculin showed mature focal adhesions (Fig. 1c, f); neither their number nor intensity was significantly affected by expression of pp41/43^{FRNK}.

As pp125^{FAK} is activated and undergoes autophosphorylation on tyrosine following integrin occupancy and cell spreading¹¹, we tested the effect of pp41/43^{FRNK} on tyrosine phosphorylation of pp125^{FAK}. Lysates were prepared from CE cells following plating on fibronectin for 20 and 60 min, and pp125^{FAK} was analysed by immunoprecipitation and immunoblotting for phosphotyrosine. Expression of pp41/43^{FRNK} significantly reduced tyrosine phosphorylation of pp125^{FAK} both at early times after plating and in cells grown continuously for up to 3 days (Fig. 3). Thus, pp41/43^{FRNK} inhibits tyrosine phosphorylation of pp125^{FAK} both during cell spreading, when the rate of increase in integrin occupancy is maximal, and in adherent cells containing mature focal adhesions.

Tensin and paxillin, two components of focal adhesions, are substrates of pp125^{FAK}, or of a kinase activated by pp125^{FAK} (ref. 12). As pp41/43^{FRNK} inhibits tyrosine phosphorylation of pp125^{FAK}, we tested whether pp41/43^{FRNK} influenced tyrosine phosphorylation of paxillin and tensin. Overexpression of pp125^{FAK} only moderately increased tyrosine phosphorylation of paxillin (Fig. 4). However, expression of pp41/43^{FRNK} significantly reduced the extent of paxillin and tensin tyrosine-phosphorylation. Co-expression of pp125^{FAK} with pp41/43^{FRNK} reversed this inhibition. Immunoblotting of paxillin and tensin confirmed that expression of pp41/43^{FRNK} did not alter expression of paxillin and tensin. Thus, the pattern of inhibition of tyrosine phosphorylation by pp41/43^{FRNK} and its rescue by co-expression of pp125^{FAK} parallels the effect of pp125^{FAK} and pp41/43^{FRNK} on cell spreading.

The observation that overexpression of pp41/43^{FRNK} inhibits tyrosine phosphorylation of pp125^{FAK}, paxillin and tensin, without apparently altering the number or morphology of focal adhesions

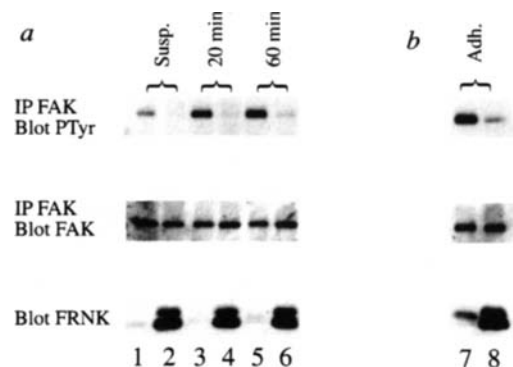


FIG. 3 Inhibition of pp125^{FAK} tyrosine phosphorylation by expression of pp41/43^{FRNK}. a, Lysates were prepared from CE cells in suspension ('Susp.') or cells that had been allowed to spread on fibronectin for the indicated times or grown in culture for 3 days ('Adh.'). pp125^{FAK} was immunoprecipitated (IP FAK) from lysates of cells infected with RCAS (lanes 1, 3, 5, 7) or with RCAS–FRNK (lanes 2, 4, 6, 8). Tyrosine phosphorylation of pp125^{FAK} was assessed by immunoblotting with anti-phosphotyrosine (PTyr) or anti-pp125^{FAK} (FAK) antibodies. Expression of pp41/43^{FRNK} was also confirmed by direct immunoblotting (FRNK) of cell extracts with the anti-pp125^{FAK} antibody. Note that expression of FRNK results in multiple bands on SDS–PAGE as a result of phosphorylation⁹ and alternative initiation of translation (A.R. and J.T.P., unpublished observations). b, Lysates from cells that had been grown in culture for 3 days after passaging and analysed as in a.

METHODS. CE cells were collected by trypsinization, allowed to spread on fibronectin-coated plastic dishes and lysates prepared after the times indicated. pp125^{FAK} was immunoprecipitated from 1.4 mg lysate protein with 20 µl of the polyclonal antiserum BC2 (BC2 is directed against the kinase domain of pp125^{FAK} and does not recognize pp41/43^{FRNK}). Immunoprecipitates were divided in two, separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with the anti-phosphotyrosine antibody RC-20 (1/2,500; Transduction Labs) or anti-pp125^{FAK} polyclonal serum BC3 (1/1,000). pp41/43^{FRNK} expression was quantified by electrophoresis of 5 µg lysate protein blotting with BC3. Blots were visualized by enhanced chemiluminescence (ECL, Amersham).

in continually adherent cells, indicates that tyrosine phosphorylation of pp125^{FAK} may not be required for the maintenance of focal adhesions. Instead, we suggest that tyrosine phosphorylation of pp125^{FAK} may be more important in regulating the formation of new focal adhesions, consistent with the observed delayed cell spreading on fibronectin (Fig. 1). This is in contrast to the results obtained with cells derived from FAK-deficient mice¹³. These cells show decreased mobility and an increased number of focal adhesions, with no apparent change in tyrosine phosphorylation of several focal adhesion proteins. The properties of FAK (–/–) cells may be due to the increased expression of focal adhesion proteins and/or increased expression of FAK-related kinases (such as PYK2/CAKβ/RAFTK), which may serve to compensate for the absence of pp125^{FAK}.

The results presented here (together with the sequence identity (99%) between pp125^{FAK} and pp41/43^{FRNK}) suggest a simple model in which pp41/43^{FRNK} inhibits the function of pp125^{FAK} by competing for a common binding partner(s) that is necessary for signalling by pp125^{FAK}. Alternatively, pp41/43^{FRNK} may act as an inhibitor of cell spreading by interacting with a protein other than a pp125^{FAK} binding protein. However, such a mechanism would imply that this protein(s) interacts only with pp41/43^{FRNK} and not pp125^{FAK}. Analysis of the structure of pp125^{FAK} has previously delimited a focal adhesion targeting sequence that is both necessary and sufficient for recruitment of pp125^{FAK} to focal adhesions¹⁴. As this sequence resides in the C-terminal domain of pp125^{FAK} and is therefore also present in pp41/43^{FRNK}, we tested whether expression of pp41/43^{FRNK} influenced the recruitment of pp125^{FAK} to focal adhesions. In cells overexpressing pp41/43^{FRNK},

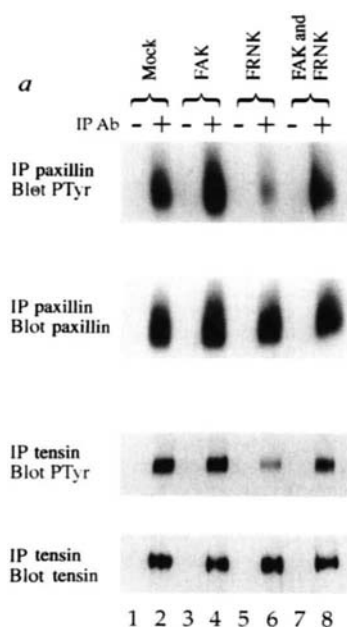
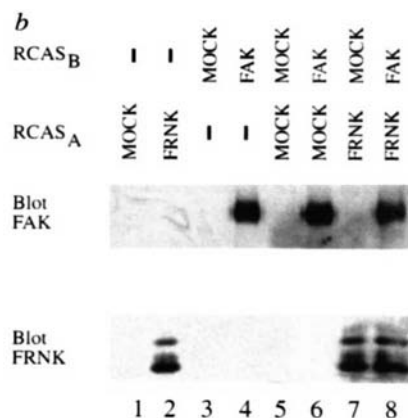


FIG. 4 Regulation of tyrosine phosphorylation of paxillin and tensin by expression of pp41/43^{FRNK} and pp125^{FAK}. **a**, Paxillin and tensin were immunoprecipitated (IP) from cells infected with RCAS wither lacking an insert (Mock; lanes 1, 2) or with RCAS-FAK (lanes 3, 4), RCAS-FRNK (lanes 5, 6), or doubly infected with both RCAS-FAK and RCAS-FRNK (lanes 7, 8). Lanes 1, 3, 5, 7 represent control immunoprecipitations in which the primary antibody was omitted. Immunoprecipitates were analysed by immunoblotting to detect phosphotyrosine (PTyr), paxillin or tensin.



b, Analysis of pp125^{FAK} and pp41/43^{FRNK} overexpression. Expression of pp125^{FAK} (lanes 4, 6, 8) and pp41/43^{FRNK} (lanes 2, 7, 8) was determined by direct immunoblotting of lysates from cells infected with the viruses indicated. Note that the amount of pp41/43^{FRNK} detected in lysates was not influenced by co-infection, indicating that the rescue of tyrosine phosphorylation of pp125^{FAK} was not due to restricting the expression of pp41/43^{FRNK}.

METHODS. CE cells were infected with RCAS containing no insert (MOCK) or RCAS-FAK (in viral subgroup B), RCAS-FRNK (in viral subgroup A), or doubly infected with RCAS-FAK and RCAS-FRNK as for Fig. 1. Cell lysates were prepared in a modified RIPA buffer, and paxillin and tensin were immunoprecipitated from 1 mg lysate protein with anti-paxillin mAb (Transduction Labs; P13520) or with the anti-tensin mAb 3C4 (ref. 22) respectively. After electrophoresis and transfer to nitrocellulose, phosphotyrosine was detected as for Fig. 1, paxillin by blotting with anti-paxillin mAb (1/10,000) and tensin by blotting with mAb 3C4 (1 µg ml⁻¹). To confirm expression of pp125^{FAK} and pp41/43^{FRNK}, 5 µg lysate was blotted directly with the polyclonal serum BC3 (1/1,000). Blots were visualized by ECL.

we did not observe a significant reduction in the intensity of staining of focal adhesions with an antiserum directed against the kinase domain of pp125^{FAK} (data not shown). Thus pp41/43^{FRNK} is unlikely to act as an inhibitor of pp125^{FAK} simply by preventing its recruitment to focal adhesions.

The regulation of pp125^{FAK} by pp41/43^{FRNK} draws attention to an emerging mechanism for regulating signal transduction. The independent expression of C-terminal binding domains has been observed with several different classes of signalling molecules, including the serine kinases myosin light-chain kinase (MLCK)^{15,16} and Ca²⁺/calmodulin-dependent kinase¹⁷, the transcription factor CREM¹⁸, and the receptor for PDGF¹⁹. Frequently, many of these domains are transcribed from a promoter within an intron immediately 5' of the exons encoding the independently expressed domain. The independently expressed domains of MLCK

(termed KRP) and CREM (termed ICER) both regulate their corresponding full-length proteins^{18,20}. To our knowledge, pp125^{FAK} provides the first example of a tyrosine kinase that is regulated by this mechanism. Further evidence that pp41/43^{FRNK} regulates FAK function in normal cells comes from data showing that pp41/43^{FRNK} is expressed endogenously in CE cells⁹, as well as rat and mouse embryo fibroblasts (A.R. and J.T.P., unpublished observations). Additionally, northern blot analysis of RNA from human²¹ and mouse (A.R. and J.T.P., unpublished observation) heart, brain and lung revealed a 2.4-kilobase FAK/FRNK RNA species comparable in size to the RNA transcript encoding chicken FRNK⁹. We suggest that the regulation of pp125^{FAK} by independently expressed pp41/43^{FRNK} may be an important mechanism for controlling the adhesive properties of cells either during development or in response to specific cellular cues. □

Received 8 December 1995; accepted 21 February 1996.

- Schaller, M. D. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 5192–5196 (1992).
- Hanks, S. K. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8487–8489 (1992).
- Kanner, S. B., Arrufo, A. & Chan, P.-Y. *Proc. natn. Acad. Sci. U.S.A.* **91**, 10484–10487 (1994).
- Lev, S. et al. *Nature* **376**, 737–745 (1995).
- Sasaki, H. et al. *J. biol. Chem.* **270**, 21206–21219 (1995).
- Abraham, S. et al. *J. biol. Chem.* **270**, 27742–27751 (1995).
- Schaller, M. D. & Parsons, J. T. *Curr. Opin. Cell Biol.* **6**, 705–710 (1994).
- Richardson, A. & Parsons, J. T. *Bioessays* **17**, 229–236 (1995).
- Schaller, M. D., Borgman, C. A. & Parsons, J. T. *Molec. cell. Biol.* **13**, 785–791 (1993).
- Nobes, C. D. & Hall, A. *Cell* **81**, 53–62 (1995).
- Schaller, M. D. et al. *Molec. cell. Biol.* **14**, 1680–1688 (1994).
- Schaller, M. D. & Parsons, J. T. *Molec. cell. Biol.* **15**, 2635–2645 (1995).

- Illic, D. et al. *Nature* **377**, 539–544 (1995).
- Hildebrand, J. D., Schaller, M. D. & Parsons, J. T. *J. Cell. Biol.* **123**, 993–1005 (1993).
- Collinge, M. et al. *Molec. cell. Biol.* **12**, 2359–2371 (1992).
- Gallagher, P. J. & Herring, B. P. *J. biol. Chem.* **266**, 23945–23952 (1991).
- Sun, Z., Sassone-Corsi, P. & Means, A. R. *Molec. cell. Biol.* **15**, 561–571 (1995).
- Molina, C. A. et al. *Cell* **75**, 875–886 (1993).
- Mosselman, S. et al. *Cancer Res.* **54**, 220–225 (1994).
- Shirinsky, V. P. et al. *J. biol. Chem.* **268**, 16578–16583 (1993).
- André, A. & Becker-André, A. *Biochem. biophys. Res. Commun.* **190**, 140–147 (1993).
- Kanner, S. B. et al. *Proc. natn. Acad. Sci. U.S.A.* **87**, 3328–3332 (1990).

ACKNOWLEDGEMENTS. We thank M. Macklem and C. Borgman for technical assistance.

CORRESPONDENCE and requests for materials should be addressed to J.T.P. (e-mail jtp@Virginia.Edu).

Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors

Yong Wan, Tomohiro Kurosaki* & Xin-Yun Huang

Department of Physiology, Cornell University Medical College,
1300 York Avenue, New York, New York 10021, USA

*Department of Oncology and Immunology, Wyeth-Ayerst Research,
Pearl River, New York 10965, USA

THE mitogen-activated protein kinase (MAPK) signalling cascade is a prominent cellular pathway used by many growth factors, hormones and neurotransmitters to regulate physiological responses^{1,2}. Although activation of the MAPK pathway by receptors with tyrosine kinase activity is well defined³, the mechanism used by heterotrimeric G-protein-coupled receptors to activate this pathway is less clear. Here we show that in cells deficient in the Src-related tyrosine kinase Lyn, stimulation of MAPK kinase and MAPK by G_q-coupled m1 muscarinic acetylcholine receptors (mAChR) is blocked, whereas G_i-coupled m2 mAChR-mediated stimulation is unaffected. In cells deficient in the tyrosine kinase Syk, both m1 and m2 mAChRs failed to stimulate MAPK kinase and MAPK. This result indicates that Syk is essential for the G_i-coupled pathway and that Lyn and Syk are necessary for the G_q-coupled pathway.

Lyn- and Syk-deficient avian B cells (DT40 cells) were generated by homologous deletion⁴. DT40 cells predominantly express Lyn among the tyrosine kinases that are closely related to Src⁴. These cells also express Syk, but not ZAP-70, among ZAP-70/Syk-family tyrosine kinases⁴. As the activation of the MAPK pathway by G-protein-coupled receptors has been extensively documented, and some of these activations are sensitive to tyrosine kinase inhibitors⁵⁻¹⁰, we have studied the involvement of tyrosine kinases in the activation of MAPK kinase (MAPKK, or MEK)¹¹⁻¹⁴, a critical protein kinase that acts upstream of MAPK and is responsible for its activation, and of MAPK by G-protein-coupled mAChRs in these tyrosine-kinase-deficient cells.

To assess the activation of MAPKK, MAPKK from DT40 cells was immunoprecipitated with a monoclonal antibody against MEK-2 (DT40 cells do not express MEK-1, the other main isoform of MAPKK) and its activity was measured by an immune-complex kinase assay^{14,15} (Fig. 1a). Immunoprecipitated MAPKK phosphorylated the purified kinase-inactive glutathione-S-transferase-MAPK fusion protein (GST-ERK-1(K63M))¹⁵ (Fig. 1b). Transient expression of G_q-coupled m1 mAChR or G_i-coupled m2 mAChR into DT40 cells stimulated (~3–4-fold) activity of MAPKK after treatment with the agonist carbachol, as reported in many other cells⁸⁻¹⁰ (Fig. 1b, c). Expression of mAChRs in transfected cells was assessed by saturation ligand-binding analysis with intact cells using the muscarinic antagonist [³H]methylscopolamine¹⁶. Although parental DT40 cells showed virtually no detectable [³H]methylscopolamine binding, cells transfected with m1 or m2 mAChRs had ~40,000 binding sites per cell. Carbachol has no effect on the basal MAPKK activity in untransfected cells. As expected, stimulation of MAPKK by m1 mAChR was insensitive to pertussis toxin, whereas stimulation by m2 mAChR was inhibited by pretreatment of the cells with pertussis toxin (data not shown). Thus, both G_q- and G_i-coupled mAChRs can activate MAPKK in DT40 cells.

In Lyn-deficient (Lyn⁻) DT40 cells, basal MAPKK activity was similar to wild-type DT40 cells (Fig. 2a). m2 mAChR increased MAPKK activity in Lyn⁻ cells to a level comparable to that induced in m2 mAChR-transfected wild-type DT40 cells (Fig. 2a). However, m1 mAChR was unable to stimulate MAPKK activity above basal level, despite the presence of similar amounts of mAChRs and MAPKK in all these cells (Fig. 2a, b). To determine whether this failure of stimulation by m1 mAChR was the result of

an absence of Lyn, we transfected wild-type Lyn back into Lyn⁻ cells. As shown in Fig. 2b, c, Lyn expression in Lyn⁻ cells increased basal MAPKK activity and, more importantly, restored the stimulation of MAPKK in response to m1 mAChR. Fyn, Lck or Src, three related tyrosine kinases that can compensate for some, if not all, Lyn functions in Lyn⁻ cells⁴, could also restore the stimulatory effect of m1 mAChR (Fig. 2c). Furthermore, both m1 and m2 mAChRs can stimulate Lyn kinase activity in wild-type DT40 cells (Fig. 2d). These results suggest that although Lyn is required for G_q-coupled m1 mAChR-mediated activation of MAPKK, it is not essential for G_i-coupled m2 mAChR-mediated activation, despite the fact that m2 mAChR can stimulate Lyn kinase activity.

We next examined the activation of MAPKK by m1 and m2 mAChRs in Syk-deficient (Syk⁻) cells. The basal MAPKK activity was similar to the wild-type DT40 cells (Fig. 3a). However, neither m1 nor m2 mAChR was able to stimulate MAPKK activity above basal levels, despite the presence of similar amounts of mAChRs and MAPKK in these cells (Fig. 3a, b). To determine whether the failure of stimulation by m1 and m2 mAChRs was a result of the

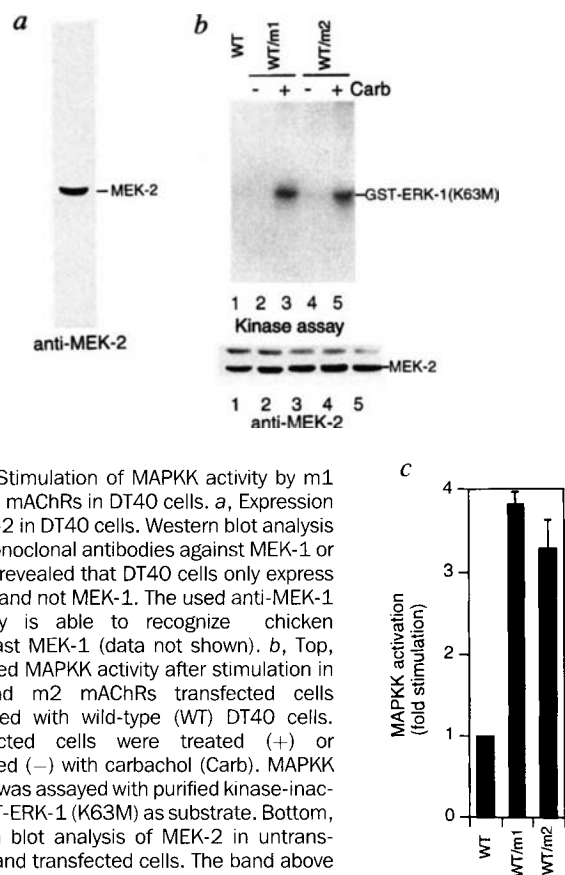


FIG. 1 Stimulation of MAPKK activity by m1 and m2 mAChRs in DT40 cells. **a**, Expression of MEK-2 in DT40 cells. Western blot analysis with monoclonal antibodies against MEK-1 or MEK-2 revealed that DT40 cells only express MEK-2 and not MEK-1. The used anti-MEK-1 antibody is able to recognize chicken fibroblast MEK-1 (data not shown). **b**, Top, Increased MAPKK activity after stimulation in m1 and m2 mAChRs transfected cells compared with wild-type (WT) DT40 cells. Transfected cells were treated (+) or untreated (-) with carbachol (Carb). MAPKK activity was assayed with purified kinase-inactive GST-ERK-1 (K63M) as substrate. Bottom, Western blot analysis of MEK-2 in untransfected and transfected cells. The band above MEK-2 is the heavy chain of IgG. **c**, Quantification of m1 and m2 mAChRs-stimulated MAPKK activity. Data represent mean $\pm$ standard deviation of six experiments.

METHODS. DT40 avian B cells were grown in RPMI supplemented with 10% heat-inactivated fetal calf serum, 1% chicken serum, 50 μ M 2-mercaptoethanol, glutamine, streptomycin and penicillin⁴. Transient transfection was done with 1 μ g plasmid DNA encoding m1 or m2 mAChR and LipofectAMINE (Gibco-BRL) in six-well culture plates¹⁶. After 24 h, transfected cells were starved in serum-free medium for 24 h and whole-cell extracts were prepared after stimulation with carbachol (1 mM) for 5 min (ref. 25). MEK-2 immunoprecipitation was done with a monoclonal antibody to MEK-2 (Transduction Labs). MAPKK was assayed as described¹⁵. (1 μ g) GST-ERK-1 (K63M) was used as substrate. After 30 min at 30 $^{\circ}$ C, samples were separated by 12% SDS-PAGE. Gels were transferred to nitrocellulose membrane filters and exposed for autoradiography. Western blots were analysed with anti-MEK-2 monoclonal antibody. Quantification was done by using a Molecular Dynamics PhosphorImager.

FIG. 2 MAPKK activity in Lyn-deficient cells. *a*, Top, although m2 increases MAPKK activity in Lyn⁻ cells, m1 mACHR fails to increase MAPKK activity above the basal level. Bottom, western blot with anti-MEK-2 antibody. The amount of MAPKK measured by western blotting was similar. *b*, Functional complementation of Lyn⁻ cells by Lck, Lyn, Src and Fyn. Expression of Lck, Lyn, Src or Fyn restored the response of Lyn⁻ cells to m1 mACHR to increase MAPKK activity. *c*, Quantification of m1 and m2 mACHRs-stimulated MAPKK activity. Values shown represent means $\pm$ s.d. of four experiments. *d*, Top, stimulation of Lyn kinase activity by m1 and m2 mACHRs. Lyn was immunoprecipitated from carbachol treated (+) or untreated (-) cells and the autophosphorylation kinase activity assay²⁶. Bottom, western blot showing similar amounts of Lyn in each lane.

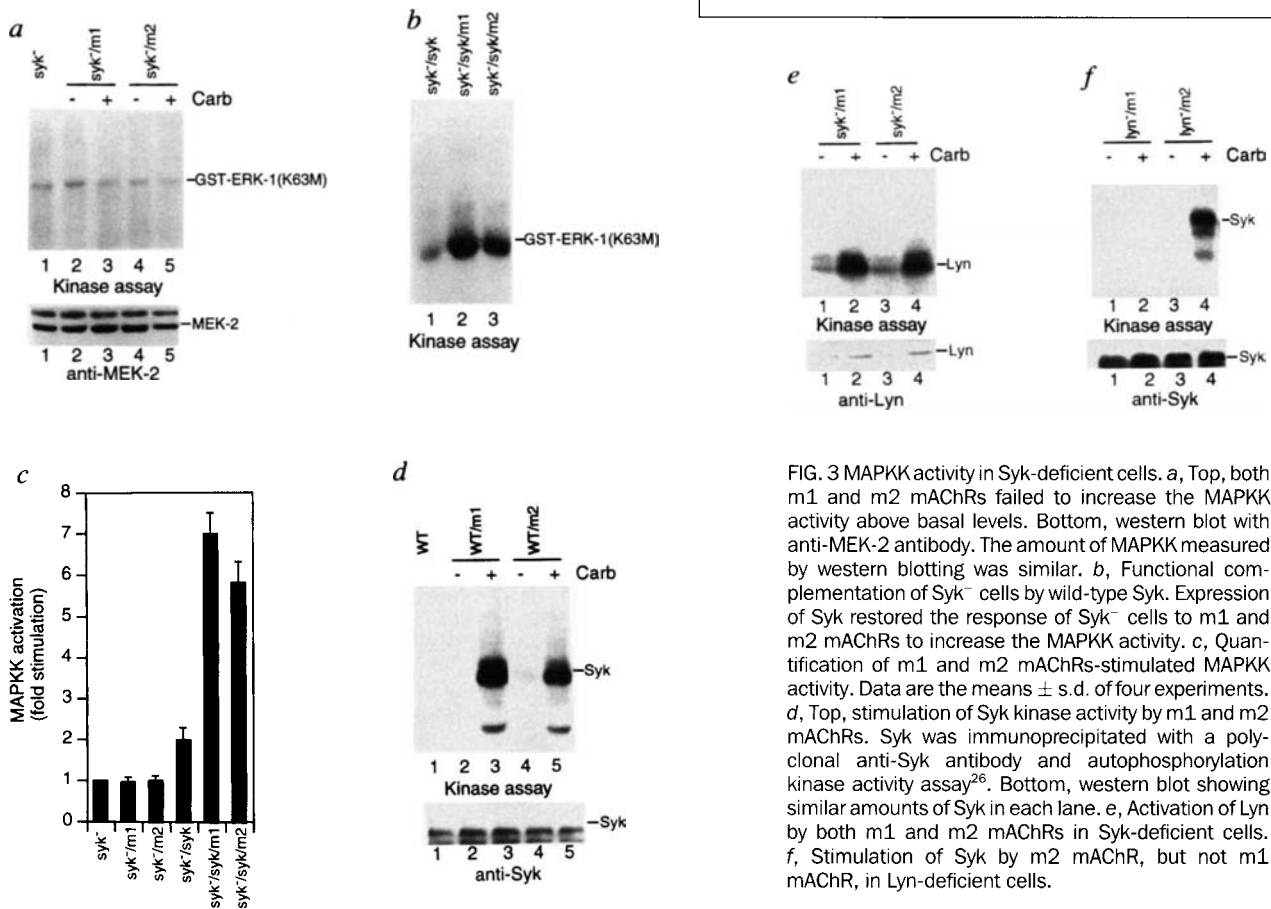
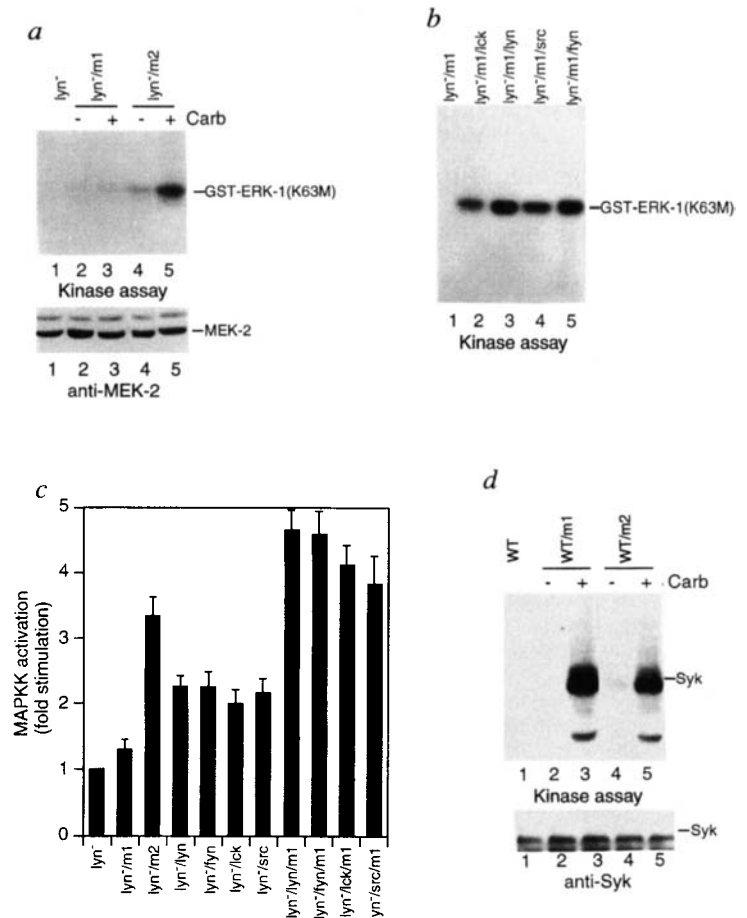


FIG. 3 MAPKK activity in Syk-deficient cells. *a*, Top, both m1 and m2 mACHRs failed to increase the MAPKK activity above basal levels. Bottom, western blot with anti-MEK-2 antibody. The amount of MAPKK measured by western blotting was similar. *b*, Functional complementation of Syk⁻ cells by wild-type Syk. Expression of Syk restored the response of Syk⁻ cells to m1 and m2 mACHRs to increase the MAPKK activity. *c*, Quantification of m1 and m2 mACHRs-stimulated MAPKK activity. Data are the means $\pm$ s.d. of four experiments. *d*, Top, stimulation of Syk kinase activity by m1 and m2 mACHRs. Syk was immunoprecipitated with a polyclonal anti-Syk antibody and autophosphorylation kinase activity assay²⁶. Bottom, western blot showing similar amounts of Syk in each lane. *e*, Activation of Lyn by both m1 and m2 mACHRs in Syk-deficient cells. *f*, Stimulation of Syk by m2 mACHR, but not m1 mACHR, in Lyn-deficient cells.

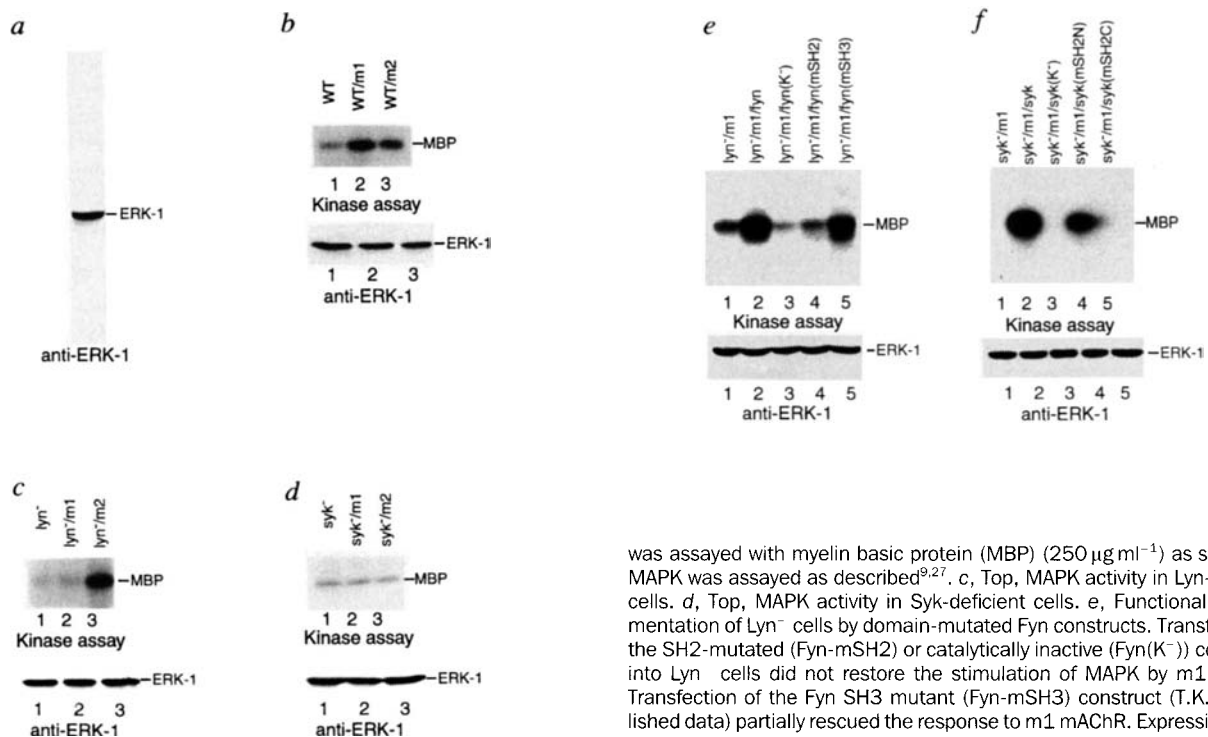


FIG. 4 Stimulation of MAPK activity by m1 and m2 mAChRs in DT40 cells. *a*, Expression of ERK-1 in DT40 cells. Western blot analysis using monoclonal antibodies against ERK-1 or ERK-2 revealed that DT40 cells only express ERK-1 and not ERK-2. The anti-ERK-2 antibody is able to recognize chicken ibroblast ERK-2 (data not shown). *b*, Top, increased MAPK activity in m1 and m2 mAChRs transfected cells. The MAPK activity

was assayed with myelin basic protein (MBP) ($250 \mu\text{g ml}^{-1}$) as substrate. MAPK was assayed as described²⁷. *c*, Top, MAPK activity in Lyn-deficient cells. *d*, Top, MAPK activity in Syk-deficient cells. *e*, Functional complementation of Lyn⁻ cells by domain-mutated Fyn constructs. Transfection of the SH2-mutated (Fyn-mSH2) or catalytically inactive (Fyn(K⁻)) constructs into Lyn⁻ cells did not restore the stimulation of MAPK by m1 mAChR. Transfection of the Fyn SH3 mutant (Fyn-mSH3) construct (T.K., unpublished data) partially rescued the response to m1 mAChR. Expression of Fyn and its mutants was similar (data not shown). *f*, Functional complementation of Syk⁻ cells by domain-mutated Syk constructs. Both C-terminal-SH2 (Syk-mSH2(C)) and kinase-domain (Syk(K⁻)) mutants could not reconstitute the response to m1 mAChR. The N-terminal-SH2-domain mutant (Syk-mSH2(N)) partially restored the responses to m1 mAChR. Expression of Syk and its mutants was similar (data not shown). In *b-f*, bottom panels show western blot analysis of MAPK in untransfected and transfected cells. Data are representative of four similar experiments.

absence of Syk, we transfected wild-type Syk back into Syk⁻ cells¹⁷. As shown in Fig. 3c, reconstitution of Syk increased basal MAPKK activity and restored the stimulation of MAPKK in response to m1 and m2 mAChRs. Furthermore, both m1 and m2 mAChRs can stimulate Syk kinase activity in wild-type DT40 cells (Fig. 2d). These results show that activation of MAPKK by both m1 and m2 mAChRs requires Syk.

In Syk⁻ cells, both m1 and m2 mAChRs increased Lyn kinase activity (Fig. 3e). In Lyn⁻ cells, m2 mAChR stimulated Syk kinase but m1-mAChR-mediated stimulation of Syk was greatly attenuated (Fig. 3f). Therefore it is likely that Lyn acts upstream of Syk in m1 mAChR signalling. The results are also consistent with our finding that Lyn is not required for Syk activation in the m2 mAChR pathway.

To investigate whether the observed increase in MAPKK activity is correlated with an increase in MAPK (ERK) activity, we tested MAPK activity in an immune-complex kinase assay using myelin basic protein (MBP) as substrate (Fig. 4). Western blot analysis showed that DT40 cells only express ERK-1, but not ERK-2 (ref. 18) (Fig. 4a). As shown in Fig. 4b, both m1 and m2 mAChRs stimulate MAPK activity in DT40 cells. Like MAPKK stimulation, MAPK stimulation by m1 mAChR is blocked in Lyn⁻ cells, whereas m2 mAChR regulation of MAPK is again not affected (Fig. 4c). In Syk⁻ cells, both m1 and m2 mAChRs failed to stimulate MAPK (Fig. 4d). Thus these tyrosine kinases are required not only for MAPKK activation but also for MAPK stimulation.

Both Lyn and Syk tyrosine kinases have several structural domains such as SH2, SH3 and kinase domains. These domains play integral parts in various aspects of signal transmission¹⁹. To test the functional importance of these domains on the mAChR-MAPK pathway, we did rescue experiments with domain-mutated

tyrosine kinases. As Fyn could compensate for Lyn's function in the m1 mAChR-MAPK signalling, we tested Fyn mutants with dysfunctional point-mutated SH2, SH3 or kinase domains²⁰. Transfection of Fyn SH2 or catalytically inactive mutant constructs into Lyn⁻ cells did not restore the stimulation of MAPK by m1 mAChR (Fig. 4e). Transfection of the Fyn SH3 mutant construct, however, partially rescued the response to m1 mAChR (Fig. 4e). These data indicate that both SH2 and kinase domains are essential for coupling m1 mAChR to MAPK and that, although the SH3 domain is not essential, its presence enhances the response.

We also tested Syk mutants with dysfunctional kinase or N- or C-terminal SH2 domains²¹. Neither the C-terminal-SH2- nor kinase-domain mutants of Syk could reconstitute the response to either m1 mAChR (Fig. 4f) or m2 mAChR (data not shown), whereas the N-terminal SH2 domain mutant of Syk partially restored the responses to m1 and m2 mAChRs (Fig. 4f). These results indicate that the association of Syk with a tyrosine-phosphorylated molecule(s), as well as its kinase activity, is essential for MAPK stimulation. The more critical requirement of the C-terminal SH2 domain of Syk is consistent with the observation that the C-terminal SH2 domain binds with greater affinity to phosphotyrosine peptide than does the N-terminal SH2 domain²².

Taken together, our results suggest that tyrosine kinases are essential in the activation pathway from G-protein-coupled receptors to MAPK in DT40 cells. Although Syk kinase is essential for the G_i-coupled m2 mAChR-MAPK pathway, both Lyn and Syk are necessary for the G_q-coupled m1 mAChR-MAPK pathway. Although G_i-mediated activation of the MAPK pathway has been shown to be sensitive to tyrosine-kinase inhibitors in some cells (such as COS-7, Rat-1, CCL39 and CHO cells) but not in others (such as Rat-1a)^{4,5,23,24}, our finding that G_q-mediated activation of

the MAPK pathway is tyrosine-kinase dependent has not been reported in other cells. This cell-type specificity of signalling pathways is more likely to be a common theme rather than a variation. □

Received 2 November 1995; accepted 29 February 1996.

1. Marshall, C. J. *Curr. Opin. Genet. Dev.* **4**, 82–89 (1994).
2. Seger, R. & Krebs, E. G. *FASEB J.* **9**, 726–735 (1995).
3. Schlessinger, J. & Ullrich, A. *Neuron* **9**, 383–391 (1992).
4. Takata, M. et al. *EMBO J.* **13**, 1341–1349 (1994).
5. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L. & Moolenaar, W. H. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1257–1261 (1993).
6. Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M. & Lefkowitz, R. J. *J. biol. Chem.* **270**, 17148–17153 (1995).
7. Cook, S. J., Rubinfeld, B., Albert, I. & McCormick, F. *EMBO J.* **12**, 3475–3485 (1993).
8. Crespo, P., Xu, N., Simonds, W. F. & Guitkin, J. S. *Nature* **369**, 418–420 (1994).
9. Faure, M., Voino-Yasenetskaya, T. A. & Bourne, H. R. *J. biol. Chem.* **269**, 7851–7854 (1994).
10. Koch, W. J., Hawes, B. E., Allen, L. F. & Lefkowitz, R. J. *Proc. natn. Acad. Sci. U.S.A.* **91**, 12706–12710 (1994).
11. Matsuda, S. et al. *EMBO J.* **11**, 973–982 (1992).
12. Seger, R. et al. *J. biol. Chem.* **267**, 14373–14381 (1992).
13. Crews, C. M., Alessandrini, A. & Erikson, R. L. *Science* **258**, 478–480 (1992).
14. Zheng, C. F. & Guan, K. L. *J. biol. Chem.* **268**, 11435–11439 (1993).
15. Alessandrini, A., Crews, C. M. & Erikson, R. L. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8200–8204 (1992).
16. Peralta, E. G. et al. *EMBO J.* **6**, 3923–3929 (1987).
17. Taniguchi, T. et al. *J. biol. Chem.* **266**, 15790–15796 (1991).
18. Boulton, T. G. et al. *Science* **249**, 64–67 (1990).
19. Cohen, G. B., Ren, R. & Baltimore, D. *Cell* **80**, 237–248 (1995).
20. Takata, M. & Kurosaki, T. *FEBS Lett.* **374**, 407–411 (1995).
21. Kurosaki, T. et al. *J. exp. Med.* **182**, 1815–1823 (1995).
22. Shiue, L. et al. *Molec. cell. Biol.* **15**, 272–281 (1995).
23. van Biesen, T. et al. *Nature* **376**, 781–784 (1995).
24. Winitz, S. et al. *J. biol. Chem.* **268**, 19196–19199 (1993).
25. Huang, X.-Y., Moricelli, A. D. & Peralta, E. G. *Cell* **75**, 1145–1156 (1993).
26. Langhans-Rajasekaran, S. A., Wan, Y. & Huang, X.-Y. *Proc. natn. Acad. Sci. U.S.A.* **92**, 8601–8605 (1995).
27. Lev, S. et al. *Nature* **376**, 737–745 (1995).

ACKNOWLEDGEMENTS. We thank R. Erikson and E. Peralta and their colleagues for cDNA clones, and M. Chao, R. Erikson, M. C. Gershengorn, T. Maack, L. G. Palmer and the members of our laboratory for reading the manuscript. This work was supported by grants from the NSF, the American Heart Association, and by Cornell University Medical College. X.-Y.H. is a Cornell Scholar and a Beatrice F. Parvin Investigator of the American Heart Association New York City affiliate.

CORRESPONDENCE and requests for materials should be addressed to X.-Y.H. (e-mail: xyhuang@med.cornell.edu).

A human peptidyl–prolyl isomerase essential for regulation of mitosis

Kun Ping Lu, Steven D. Hanes* & Tony Hunter

Molecular Biology and Virology Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, USA

* Wadsworth Center, Laboratory of Developmental Genetics, New York State Department of Health, 120 New Scotland Avenue, Albany, New York 12201, USA

THE NIMA kinase is essential for progression through mitosis in *Aspergillus nidulans*^{1–6}, and there is evidence for a similar pathway in other eukaryotic cells^{5–8}. Here we describe the human protein Pin1, a peptidyl–prolyl *cis/trans* isomerase (PPIase) that interacts with NIMA. PPIases are important in protein folding, assembly and/or transport^{9–13}, but none has so far been shown to be required for cell viability^{9–11}. Pin1 is nuclear PPIase containing a WW protein interaction domain, and is structurally and functionally related to Ess1/Ptf1, an essential protein in budding yeast^{14,15}. PPIase activity is necessary for Ess1/Pin1 function in yeast. Depletion of Pin1/Ess1 from yeast or HeLa cells induces mitotic arrest, whereas HeLa cells overexpressing Pin1 arrest in the G2 phase of the cell cycle. Pin1 is thus an essential PPIase that regulates mitosis presumably by interacting with NIMA and attenuating its mitosis-promoting activity.

One class of human complementary DNA clones identified in a

yeast two-hybrid screen for proteins interacting with NIMA encoded Pin1, a protein of relative molecular mass (*M*_r) 18,245 (Fig. 1a). Northern blotting showed a single ~1.0-kilobase (kb) *PIN1* messenger RNA in all human cell lines tested (data not shown). Pin1 is 45% identical to the budding yeast protein Ess1/Ptf1 (refs 14,15), and contains a putative nuclear localization signal and two identifiable domains, an amino-terminal WW domain and a carboxy-terminal PPIase domain (Fig. 1a). The WW domain, characterized by two invariant tryptophans, occurs in a variety of unrelated cell-signalling proteins¹⁶; in Yap the WW domain mediates protein–protein interactions by binding a Pro-rich sequence¹⁷. Thus the Pin1 WW domain may act as a protein interaction module that confers specificity to its catalytic domain.

The C terminus of Pin1 contains motifs characteristic of the newly discovered parvulin family of PPIases, which share little similarity with either the cyclophilins or the FK506-binding proteins (FKBPs)^{12,13} (Fig. 1a). Recombinant Pin1 had concentration-dependent PPIase activity (Fig. 1b), which, like parvulin¹², was not inhibited by either cyclosporin A or an FK506 analogue (data not shown). Our results confirm that Pin1 belongs to this family of PPIases^{12,13}. Previous failure to detect Ess1/Ptf1 PPIase activity¹⁵ might have been due to its high sensitivity to the chymotrypsin used in the assay. With the possible exception of yeast *CPR3*, which is required under restricted conditions¹⁸, no PPIase has yet been found to be essential for growth^{9–11}. Here we show that Pin1/Ess1 is required for normal growth.

The striking sequence similarity between Pin1 and Ess1 prompted us to test whether *PIN1* substitutes for *ESS1* in yeast cells. Diploid cells bearing one disrupted copy of *ESS1* were transformed with a *PIN1* expression plasmid or a control vector, followed by tetrad analysis (Fig. 2a). As expected¹⁴, tetrads from cells transformed with the control plasmid showed 2:2 segregation for spore viability (viable:inviable). In contrast, ~25% of tetrads from *PIN1*-transformed cells showed 4:0 segregation, demonstrating that *PIN1* complements the *ess1*[–] mutation in haploid cells. The growth rates of *PIN1*-expressing *ess1*[–] cells were similar to those of control cells (Fig. 2a). In plasmid shuffle experiments, *PIN1* also complemented the lethal defect in *ess1*[–]/*ess1*[–] diploid cells (data not shown). Thus human *PIN1* substitutes functionally for *ESS1* in both haploid and diploid yeast cells.

To determine whether the PPIase activity of Pin1 is necessary for its essential function, we tested whether PPIase domain mutants complemented *ess1*[–] yeast by tetrad analysis. Cells expressing wild-type Pin1 showed 4:0 segregation for spore viability, whereas cells expressing Pin1 with a C-terminal truncation beyond Ser 114 (*PIN1*-3'Δ) or with Ala substitutions in three highly conserved residues (*PIN1*-3A; G155A, H157A, I159A) showed 2:2 segregation, indicating a failure to complement the *ess1*[–] mutation (Fig. 2b). A plasmid shuffle assay gave similar results (data not shown). Immunoblot analysis showed that Pin1 and Pin1-3'Δ were expressed at similar levels, whereas slightly less Pin1-3A was expressed (data not shown). Neither mutant protein had detectable PPIase activity when assayed *in vitro* (Fig. 2b). These results demonstrate that Pin1/Ess1 PPIase activity is required for cell growth.

To determine the cell-cycle function of *PIN1/ESS1* in yeast, we constructed a haploid *ess1*[–] strain that expressed *PIN1* under control of the *GAL1* promoter (*GAL1*–*PIN1*). Regulated expression of Pin1 was confirmed by immunoblotting (data not shown). As expected, this *PIN1*-dependent strain grew normally in inducing or partial-inducing medium but did not grow in repressing medium. Cells depleted of Pin1 displayed a striking terminal phenotype: mitotic arrest followed by nuclear fragmentation. Cell division slowed 6 hours after shifting from inducing to repressing medium, and by 12 hours cells accumulated in the M phase with 2*n* DNA content and a large bud (Fig. 2c). Most cells contained nuclei stuck in the neck between mother and bud, although tubulin staining revealed a typical mitotic spindle (Fig. 2c, middle inserts). By 24 hours, ~80% of cells were arrested in M phase; they had low Cdc28 activity and lacked Clb2 (data not

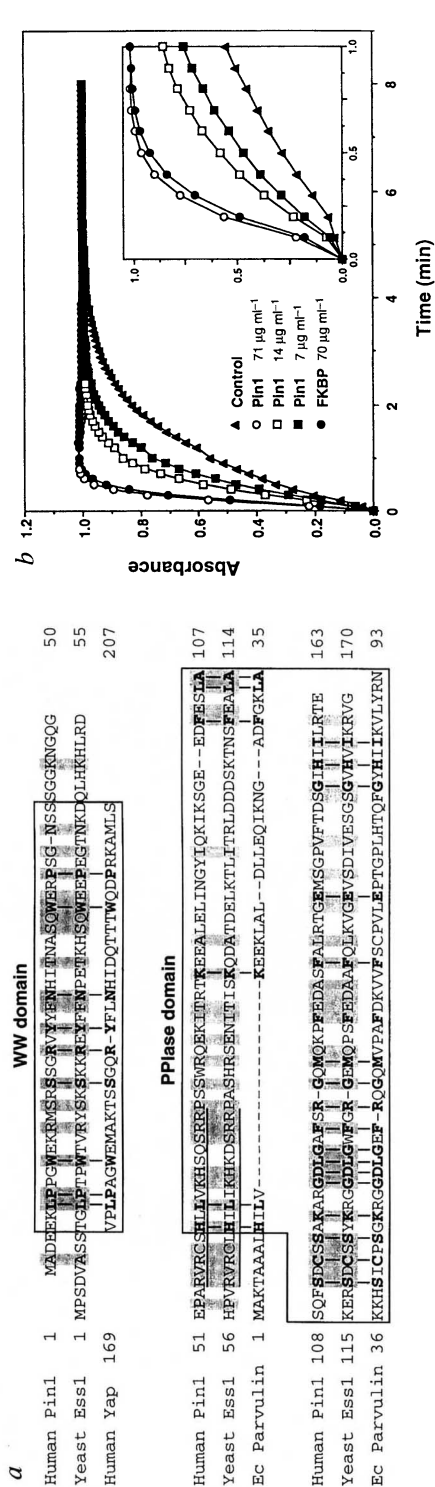


FIG. 1 The human Pin1 PPIase and its homology to Ess1, parvulin and Yap WW domain. **a**, Alignment of Pin1, Ess1, Human Yap WW domain and *Escherichia coli* parvulin. Dashes indicate gaps, vertical lines indicate identity among all three sequences, and shaded boxes indicate identity between Pin1 and Ess1. Underlined residues form a consensus bipartite nuclear localization signal. **b**, Isoenzyme activity was monitored by a change in the absorbance at 395 nm, with the final stable absorbance of each sample being set at 1.0. Different concentrations of Pin1 were used as indicated, with FKBP PPIase as a control. The insert shows PPIase activity during the first minute of assay.

METHODS. For the yeast two-hybrid screen, *Aspergillus nima* was fused to the GAL4 DNA-binding domain in the pAS2 vector²⁵. Initially, Y190 cells were transformed with the NIMA/pAS2 expression vector but failed to produce stable strains, probably because overexpression of NIMA induces premature mitotic arrest in budding yeast, as it does in other eukaryotic cells^{1,3,7,8}. Therefore NIMA was cotransformed with a HeLa cell cDNA library fused to the GAL4 activation domain²⁶, and screening was done as described²⁵. Of 10⁶ colonies screened, 13 showed a specific interaction with NIMA and belonged to three distinct gene classes, referred to as PIN1-3. Six clones contained cDNAs encoding PIN1, with different sequences between C-9 and G+15. Additional 5' sequence was obtained by screening a HeLa cell cDNA library (from R. Fukunaga). To express the (His)₆-tagged Pin1 protein, PIN1 cDNA was subcloned into pProEx1, and the recombinant protein was expressed in and purified from BL21 bacteria using Ni²⁺-NTA agarose as described by the manufacturer (Qiagen). FKBP was provided by D. Schultz and C. Zuker. Isoenzyme activity was measured as described²⁷.

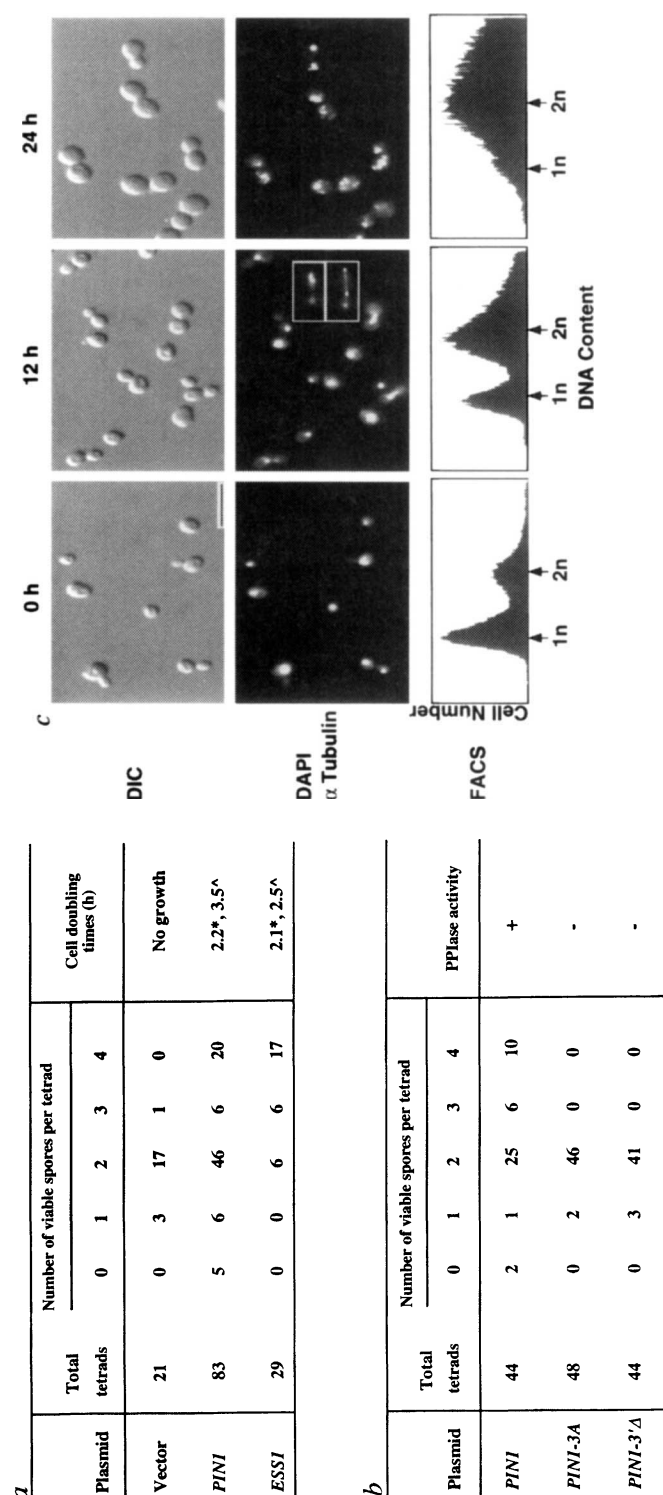
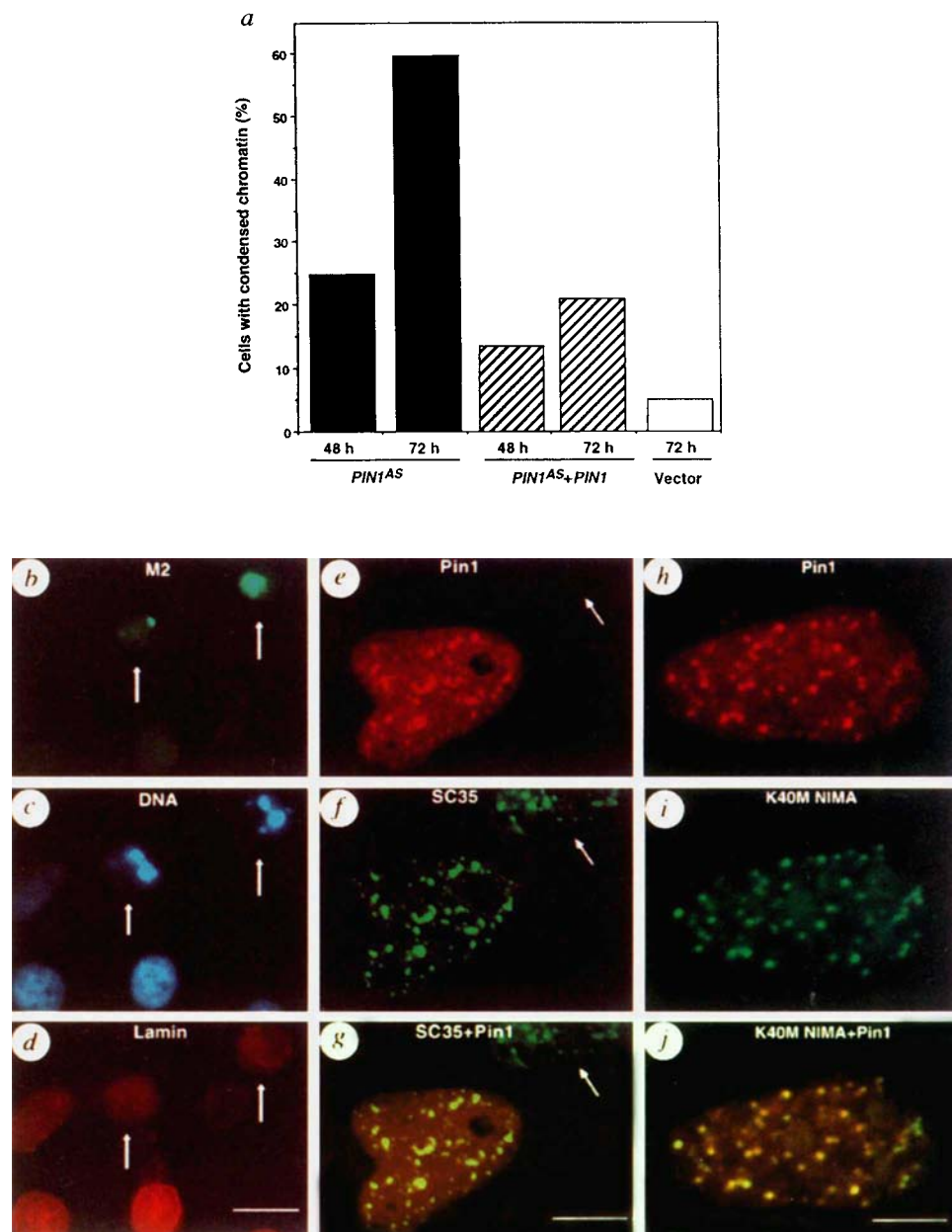


FIG. 2 PIN1 functions as a mitotic regulator in yeast and its PPIase is required for cell viability. **a**, PIN1 complements *ess1*⁻ yeast. Disruption strain MGG3/pSHU (*ess1*::URA3/ESS1)¹⁹ was transformed with vector alone, or with plasmids expressing PIN1 or ESS1. Cells were induced to sporulate, tetrads dissected, and the number of viable spores scored after 3 days. Doubling times were determined by growing PIN1- or ESS1-expressing *ess1*::URA3 segregants in rich (*) or synthetic (') media. **b**, PIN1's PPIase activity is required for function. MGG3/pSHU was transformed with plasmids expressing wild-type or PPIase mutant PIN1. Spore viability was analysed as in **a**. PPIase activity was assayed *in vitro* using a peptide substrate. **c**, Depletion of Pin1/Ess1 causes mitotic arrest and nuclear fragmentation. PIN1-dependent haploid cells (*ess1*⁻ GAL-PIN1) were shifted from inducing (galactose) to repressing (glucose) media, collected and fixed at the times indicated. Cells were stained with DAPI or anti-tubulin mAb (inset) and visualized by Nomarski (DIC) or fluorescent illumination. For FACS analysis, cells were stained with propidium iodide. Scale bar, 10 μm ; insets show a higher magnification of DAPI (top) and α -tubulin (bottom) staining of a representative cell.

METHODS. For genetic complementation, PIN1 cDNA was expressed from the constitutive yeast triose-phosphate isomerase promoter in 2 μ vector, pJK305-TPI (provided by J. Kamens and R. Brent). PPIase mutants were expressed from the GAL1 promoter in pBC103 (B. Cohen and R. Brent), and cotransformed with artificial activator GAL4-ER-VP16 (ref. 28) into MGG3/pSHU, which is *gal2*⁻. Tetrads were dissected on plates containing 5×10^{-9} M β -oestradiol. Expression and PPIase assays were done as in Fig. 1b. Tubulin staining was done as described²⁹. The Pin1-dependent *ess1*⁻ strain (YSH12) carries an integrated copy of the PIN1 cDNA under control of the GAL1 promoter.

FIG. 3 Pin1 colocalizes with NIMA in the nuclear speckle and induces mitotic arrest when underexpressed in HeLa cells. **a**, tTA-1 cells were transfected with the indicated construct plus FLAG-tagged K40M NIMA280, which does not affect the cell cycle⁷ and lacks the Pin1-interaction domain. Cells were stained with anti-FLAG tag M2 mAb and scored for cells with mitotic phenotype. In each case, at least 250 M2-positive cells were counted in random fields. **b–d**, tTA-1 cells transfected with *PIN1*^{AS} plus FLAG-tagged K40M NIMA280 for 72 h were triply stained for transfected cells (b) using M2 mAb, for DNA (c) using Hoechst, and for the nuclear lamina (d) using anti-lamin A polyclonal antibodies. The arrows point to transfected cells identified by M2 staining for K40M NIMA280, which is localized in the Golgi complex. Scale bar, 5 μ m. **e–j**, tTA-1 cells transfected for 24 h with the vectors expressing HA-tagged Pin1 alone (e–g), or plus FLAG-tagged kinase-negative K40M NIMA (h–j), were doubly stained for Pin1 using anti-HA tag 12CA5 mAb (IgG2b) and for SC35 using anti-SC35 mAb (IgG1) or NIMA using anti-FLAG tagged M2 mAb (IgG1). The two different antibody isotypes were probed with anti-IgG2b-Texas Red and anti-IgG1-FITC, respectively, followed by confocal microscopy. Double-staining for Pin1 and SC35 or NIMA was displayed by superimposing the respective images, with yellow indicating colocalization. Pin1 was also colocalized with wild-type NIMA in NIMA-induced mitotic cells (data not shown). Arrows point to an untransfected cell, indicating very little crossreactivity among the antibodies. Scale bar, 1 μ m. **METHODS.** *PIN1* cDNA was subcloned into the pUHD-P2 vector so that it was expressed with a single N-terminal HA tag or into the pUHD-P1 vector in the anti-sense orientation (*PIN1*^{AS}), transfected into tTA-1 cells (a HeLa-derived cell line), followed by indirect fluorescence microscopy, as described⁷.



shown), indicating that the arrest point was after cyclin B (Cib2) degradation. Arrested cells showed extensive nuclear fragmentation, accompanied by collapse of the mitotic spindle; this began at ~18 hours, and was essentially complete by 24 hours (Fig. 2c; data not shown). As expected, these phenotypes appeared earlier in cells that expressed lower initial Pin1 concentrations (data not shown). Thus depletion of Pin1/Ess1 induces mitotic arrest and eventual nuclear fragmentation, suggesting that Pin1/Ess1 is required to inhibit entry into M and/or to promote exit from M.

To examine Pin1 function in human cells, haemagglutinin (HA)-tagged *PIN1* and/or antisense full-length *PIN1* (*PIN1*^{AS}) was expressed in HeLa cells, followed by fluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis, as described⁷. About 50% of cells overexpressing Pin1 arrested in G2, whereas control cells not expressing Pin1 (within the Pin1-transfected population) and vector-transfected cells displayed a normal cell-cycle profile (data not shown), indicating that overexpression of Pin1 inhibits the G2/M transition. In contrast, cells transfected with *PIN1*^{AS} displayed mitotic phenotypes including cell rounding, chromatin condensation and nuclear-lamin disassembly (Fig. 3b–d). These phenotypes appeared 48 hours after transfection,

and were evident in ~60% of cells by 72 hours, when the nucleus became fragmented in most cells. The *PIN1*^{AS}-induced mitotic phenotype was dramatically reduced by coexpression of sense *PIN1* cDNA. Because depletion of Pin1 in yeast also induced a similar phenotype, these results indicate that Pin1 and Ess1 have a highly conserved mitotic function.

The mitotic function of Pin1 prompted us to examine its subcellular localization. Pin1 was almost exclusively nuclear, with a speckled appearance (Fig. 3e–g). These speckles were identical to those detected using anti-SC35 splicing factor monoclonal antibody¹⁹ (Fig. 3e–g). Thus Pin1 is associated with the nuclear speckle, which is a key component of the nuclear scaffold or matrix and undergoes disassembly in mitosis²⁰; several other mitosis-related proteins are also associated with this structure. The fission yeast protein CDC28, required for M phase, is a splicing factor²¹. The yeast Dsk1 kinase, a suppressor of the mitotic *dis1* mutant²², is homologous to a human mitotically active splicing factor kinase, Srp1 (ref. 23), and overexpression of Dsk1 delays the G2/M transition²². These results suggest that the reorganization of the nuclear speckle may be needed for entry into M phase.

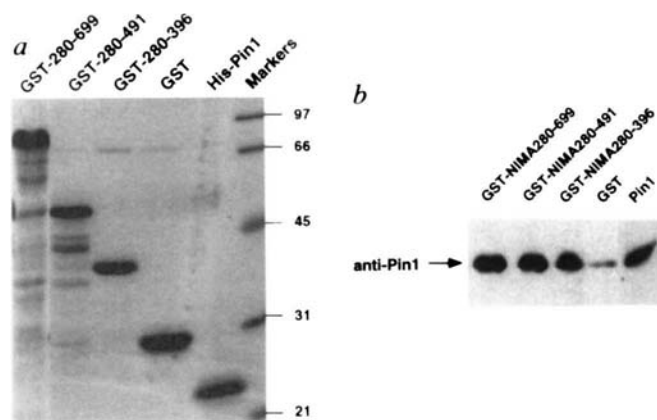
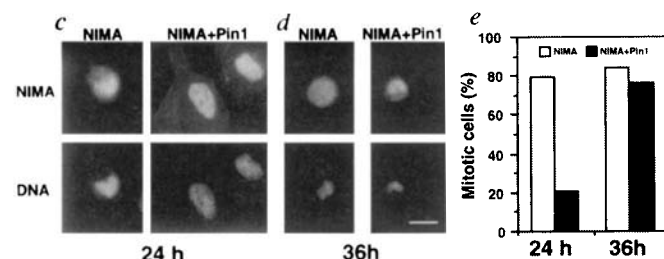


FIG. 4 Pin1 interacts with the NIMA essential protein-interacting domain (NID) and attenuates its mitosis-promoting activity when overexpressed in HeLa cells. *a, b*, Recombinant purified His-Pin1 and different glutathione S-transferase (GST)-NIMA fusion proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining (*a*). GST-NIMA beads were incubated with His-Pin1 for 2 h and washed extensively. Pin1 bound to the beads was detected by Coomassie staining, followed by immunoblot analysis using Pin1-specific polyclonal antibodies (*b*). Based on Coomassie staining, about one-fifth of the Pin1 input was precipitated by each GST-NIMA protein. *c-e*, tTA-1 cells were cotransfected with FLAG-NIMA and HA-Pin1



or control vector for 24 (*c*) or 36 h (*d*) and stained with M2 mAb and Hoechst dye to examine the phenotype of NIMA-expressing cells under a fluorescence microscope. *e*, The percentage of cells with rounding and chromatin condensation scored in a sample of at least 250 M2-positive cells. Scale bar, 5 μ m.

METHODS. *a, b*, The PIN1 cDNA was subcloned into pET28a and different domains of NIMA⁷ into a pGEX vector and the recombinant proteins were purified from bacteria using Ni²⁺-NTA or glutathione-agarose column, as described³⁰. *In vitro* binding was performed at 4 °C for 2 h in NP40 buffer (50 mM Tris-HCl, pH 8.0, 0.5% NP40, 200 mM NaCl, 20 mM β -glycero-phosphate, 20 mM NaF, 0.1 mM sodium orthovanadate, 50 μ g ml⁻¹ phenylmethylsulphonyl fluoride, 10 μ g ml⁻¹ leupeptin, 10 μ g ml⁻¹ aprotinin) containing 1 mg ml⁻¹ BSA, followed by washing 8 times with the buffer without BSA. Pin1 was detected by immunoblot analysis using Pin1 antibodies, which were generated in rabbits against the C-terminal 17 residues. The antibodies specifically recognised an 18K endogenous Pin1 protein in HeLa cell lysates. *c-e*, tTA-1 cells were cotransfected with FLAG-NIMA and HA-PIN1 or vector for 24 or 36 h, followed by fluorescence microscopy, as described⁷.

Manipulation of Pin1 or NIMA functions induces strikingly similar, albeit reciprocal, phenotypes, suggesting that they are in a common pathway. Dominant-negative mutant NIMA induces G2 arrest, whereas NIMA overexpression induces M arrest and nuclear fragmentation^{1,3,5,8}, with the arrest point being after cyclin B degradation if overexpressed in G2 (ref. 4). Pin1 specifically interacts with the NIMA C-terminal regulatory domain in the two-hybrid system (data not shown). *In vitro* assays using recombinant proteins indicated that, although Pin1 did not act as a substrate or a inhibitor of NIMA kinase (data not shown), it specifically interacted with residues 280–396 of NIMA (Fig. 4*a, b*), which has previously been shown to be essential for NIMA *in vivo* function and proposed to be a protein-interacting domain (NID)^{5,7}. Furthermore, Pin1 and NIMA co-immunoprecipitated (data not shown) and colocalized in nuclear speckles in HeLa cells (Fig. 3*h-j*). Moreover, coexpression of Pin1 and NIMA attenu-

ated NIMA mitosis-promoting activity (Fig. 4*c-e*). In addition, Pin1 was originally isolated by its ability to suppress the NIMA lethal phenotype in yeast (Fig. 1*a* legend). These results indicate that Pin1 binds the NID domain of NIMA and somehow inhibits its mitotic function. Because activation and inactivation of NIMA are required for entry into and exit from M, respectively^{2,4,7}, it is possible that Pin1 is involved in regulation of both these checkpoints, although we do not know whether Pin1 also has another role in exit from M.

Thus Pin1, a novel conserved PPIase, is an essential mitotic regulator, presumably in the NIMA pathway. Pin1/Ess1 is the first reported PPIase involved in cell-cycle control. The recent identification of a Pin1/Ess1 homologue in *Drosophila*, *dodo*, which functions in yeast²⁴, underscores the conserved nature of this protein. By using Pin1, it may be possible to identify functional homologues of NIMA in vertebrates. □

Received 23 January; accepted 20 February 1996.

- Osmani, S. A., Pu, R. T. & Morris, N. R. *Cell* **53**, 237–244 (1988).
- Osmani, A. H., McGuire, S. L. & Osmani, S. A. *Cell* **67**, 283–291 (1991).
- Lu, K. P. & Means, A. R. *EMBO J.* **13**, 2103–2113 (1994).
- Pu, R. T. & Osmani, S. A. *EMBO J.* **14**, 995–1003 (1995).
- Lu, K. P. & Hunter, T. in *Progress in Cell Cycle Research* Vol. 1 (eds Meijer, L., Guidet, S. & Tung, H. Y. L.) 187–205 (Plenum, New York, 1996).
- Fry, A. M. & Nigg, E. A. *Curr. Biol.* **5**, 1122–1125 (1995).
- Lu, K. P. & Hunter, T. *Cell* **81**, 413–424 (1995).
- O'Connell, M. J., Norbury, C. & Nurse, P. *EMBO J.* **13**, 4926–4937 (1994).
- Schreiber, S. L. *Science* **251**, 283–287 (1991).
- Heitman, J., Movva, N. R. & Hall, M. N. *New Biol.* **4**, 448–460 (1992).
- Fischer, G. *Angew. Chem. int. Edn. engl.* **33**, 67–118 (1994).
- Rahfeld, J. U. et al. *FEBS Lett.* **343**, 65–69 (1994).
- Rudd, K. E. et al. *Trends biochem. Sci.* **20**, 12–14 (1995).
- Hanes, S. D., Shank, P. R. & Bostian, K. A. *Yeast* **5**, 55–72 (1989).
- Hani, J., Stumpf, G. & Domdey, H. *FEBS Lett.* **365**, 198–202 (1995).
- Sudol, M. et al. *FEBS Lett.* **369**, 67–71 (1995).
- Chen, H. I. & Sudol, M. *Proc. natn. Acad. Sci. U.S.A.* **92**, 7819–7823 (1995).
- Davis, E. S. et al. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11169–11173 (1992).
- Fu, X. D. & Maniatis, T. *Nature* **343**, 437–441 (1990).
- Spector, D. L. *A. Rev. Cell Biol.* **9**, 265–315 (1993).

- Fu, X. D. *RNA* **1**, 663–680 (1995).
- Takeuchi, M. & Yanagida, M. *Molec. Biol. Cell* **4**, 247–260 (1993).
- Gui, J. F., Lane, W. S. & Fu, X. D. *Nature* **369**, 678–682 (1994).
- Maleszka, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **93**, 447–451 (1996).
- Harper, J. W. et al. *Cell* **75**, 805–816 (1993).
- Hannon, G. J., Demetrick, D. & Beach, D. *Genes Dev.* **7**, 2378–2391 (1993).
- Heitman, J. et al. *Methods* **5**, 176–187 (1993).
- Louvion, J. F., Havaux, C. B. & Picard, D. *Gene* **131**, 129–134 (1993).
- Roberts, C. J. et al. *Meth. Enzym.* **194**, 644–661 (1991).
- Lu, K. P., Osmani, S. A. & Means, A. R. *J. biol. Chem.* **268**, 8769–8776 (1993).

ACKNOWLEDGEMENTS. We thank X. D. Fu, J. Heitman, S. Forsburg, M. Latterich, J. Noel, H. Toyoshima and D. Nag for advice; G. Hannon, D. Beach, S. Elledge, X. D. Fu, R. Fukunaga, L. Gerace, M. D. Mendenhall and H. Bujard for reagents; D. Schultz and C. Zuker for help with the PPIase assay; R. Hackett for help with some yeast experiments; M. Latterich and G. Mondesert for help with tubulin staining; T. Murray, G. Karpen and R. Glaser for help with microscopy; V. Edelman and T. Deerinck for help with confocal microscopy; D. Chambers for help with FACS analysis; and Wadsworth Center Core Facilities for microscopy and oligonucleotide synthesis. The studies were supported by grants from Health Research Inc. (S.D.H.) and the USPHS (T.H. and M. Ellisman). K.P.L. is a Leukemia Society of America fellow, and T.H. is an American Cancer Society research professor.

CORRESPONDENCE and requests for materials should be addressed to K.P.L. (e-mail: Lu@SC2.Salk.Edu). The GenBank accession number for Pin1 is U49070.

Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2

Pierre Colas, Barak Cohen, Timm Jessen*, Irina Grishina, John McCoy† & Roger Brent

Department of Molecular Biology, Massachusetts General Hospital, 50 Blossom Street, Boston, Massachusetts 02114, and Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

† Genetics Institute Inc., 89 Cambridge Park Drive, Cambridge, Massachusetts 02142, USA

* Present address: Hoechst Marion Roussel, Building H825, D-65926 Frankfurt, Germany.

A NETWORK of interacting proteins controls the activity of cyclin-dependent kinase 2 (Cdk2) (refs 1, 2) and governs the entry of higher eukaryotic cells into S phase. Analysis of this and other genetic regulatory networks would be facilitated by intracellular reagents that recognize specific targets and inhibit specific network connections. We report here the expression of a combinatorial library of constrained 20-residue peptides displayed by the active-site loop of *Escherichia coli* thioredoxin, and the use of a two-hybrid system to select those that bind human Cdk2. These peptide aptamers were designed to mimic the recognition function of the complementarity-determining regions of immunoglobulins. The aptamers recognized different epitopes on the Cdk2 surface with equilibrium dissociation constant in the nanomolar range; those tested inhibited Cdk2 activity. Our results show that peptide aptamers bear some analogies with monoclonal antibodies, with the advantages that they are isolated together with their coding genes, that their small size should allow their structures to be solved, and that they are designated to function inside cells.

We based our approach on the fact that peptide loops that are anchored at both their amino and carboxy termini, such as those

found in antibody and T-cell receptor complementarity-determining regions, are capable of specific and high-affinity molecular recognition. The active-site loop of *E. coli* thioredoxin (TrxA) can be used as a scaffold to display such conformationally constrained peptides³, which, when expressed as fusions to the flagellin gene product, can recognize distinct combinations of shape, charge and hydrophobicity⁴. Moreover, TrxA is small, soluble, rigid and readily expressed and purified from *E. coli*.

We constructed a library that directed the synthesis in yeast of constrained peptides of random sequence, displayed in the active site of *E. coli* TrxA and fused to a modified set of protein moieties from the original interaction trap⁵, a yeast two-hybrid system (Fig. 1a). We chose 20 residues (20-mers) for the length of the displayed peptides, which is long enough to fold into many patterns of shape and charge, but sufficiently short that many of their encoding oligonucleotides lacked stop codons and to enable the structure of the encoded proteins to be easily solved. The library contained 2.9×10^9 members, of which $>10^9$ directed the synthesis of peptides. Because of the presence of fortuitous restriction sites in some coding oligonucleotides and because some library members contained double inserts, about one fifth of the constrained peptides were longer or shorter than unit length. We introduced this interaction library into a selection strain containing a LexA-Cdk2 bait. From 6.0×10^6 transformants, we isolated 14 plasmids that expressed peptide aptamers that interacted with Cdk2 but not with control proteins (Fig. 1b).

We used an interaction mating assay⁶ to examine the strength and specificity with which the peptide aptamers bound Cdk2 (Fig. 2a). As judged by the blue colour on interaction matrix plates containing X-gal, all 14 aptamers interacted with the LexA-Cdk2 bait but not with unrelated proteins such as Max or Rb, or with certain CDK family members such as Cdk4, which shares 47% sequence identity with Cdk2. However, some aptamers interacted with other CDK family members. The fact that different peptide aptamers showed distinct patterns of crossreactivity with different CDKs indicated that these aptamers recognized different epitopes conserved among different subsets of CDKs. The sequence of the variable regions is shown in Fig. 2b. Sequences that were not of unit length occurred at the same frequency among the Cdk2-interacting aptamers as in the library as a whole. No variable region showed notable sequence similarity to known proteins, as would be expected if the 20-mer peptides

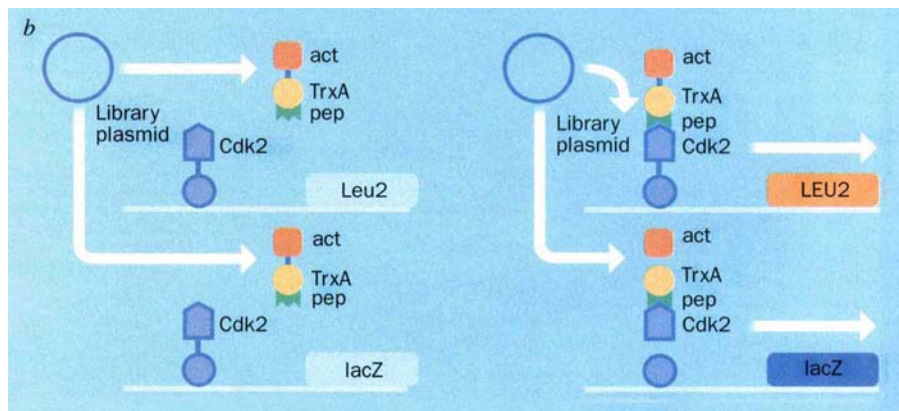
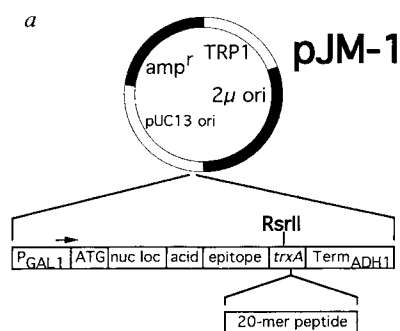
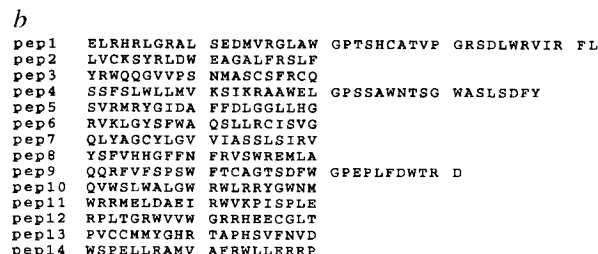


FIG. 1 a, Peptide aptamer expression vector. b, Selection of anti-Cdk2 aptamers.

METHODS. a, We used the oligonucleotides 5'-CAGTCAGTCAGTCAATTGAA-GAAGGAGATATACATATGGGTGCTCCTCCAAAAAGAGAAAGGTAGCTGGT-TCTGAGTCCCGGGATCACCTTGGGATTGAGGA-3' and 5'-ACTGACTGACTG-CATATGGAATTCAGAGGCATAATCTGGCACATCATAAGGGTAGGACCCAAACAA-AGGTCTGTTCCGCCTGAGTGACGTTAGCAGCAGGAAGTACCGGATGACCGCC-TTTCGCAACGG-3' to generate a PCR fragment from pLexA-B112²¹ encoding a fusion between the SV40 nuclear localization sequence, the B112 'acid blob', and the haemagglutinin epitope tag, flanked by *MunI* and *NdeI* sites. We excised *trxA* from pALTRXA-781³ with *NdeI* and *Sall*. We

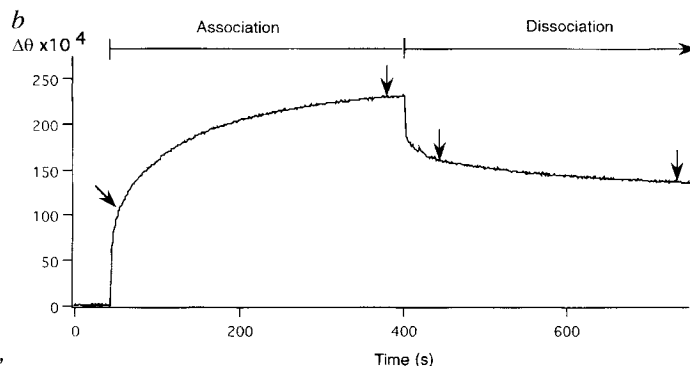
introduced both fragments into the *EcoRI*-*XhoI*-cut pJG4-4⁵ to create pJM-1. We made the library by annealing 1 nmol oligonucleotide 5'-GACTGACTGGTCCG(NNG/T)₂₀GGTCTCAGTCAGTCAG-3' to 1 nmol 5'-CTG-ACTGACTGAGGACC-3', synthesizing the second strand with Klenow, cutting the product with *Avall*, ligating it into *RsrII*-cut pJM-1, and transforming *E. coli* GL724³ to get 2.7×10^9 transformants. b, We transformed 100 μg of the library²² into EGY48 containing the LexAop-lacZ reporter pSH18-34²³ and a LexA-Cdk2 bait⁵. We isolated interactors from 6×10^7 cells derived from 6×10^6 transformants⁵. Sixty-six colonies were galactose-dependent LEU⁻ and blue on X-gal. Library plasmids from these colonies contained 14 different sequences.



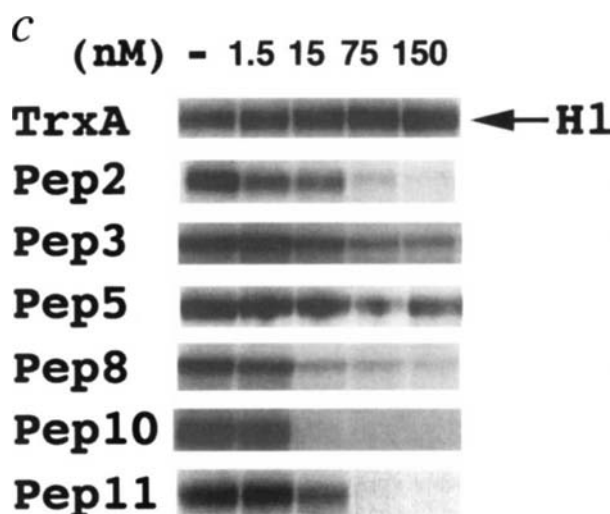
METHODS. *a*, We transformed EGY48 with plasmids expressing the different anti-Cdk2 aptamers and one that contained a control 20-mer peptide loop, and then mated these transformants to different bait strains as described⁶. *b*, We sequenced the DNA encoding the peptide loops using a dideoxy kit (USB).

We arbitrarily chose six aptamers for further study. The peptide aptamers interacted with a glutathione *S*-transferase (GST)–Cdk2 fusion protein *in vitro* (Fig. 3a), demonstrating that these interac-

tions were independent of any bridge proteins native to yeast. On the basis of interpolation from interaction trap calibration experiments⁷, the robust transcription that some aptamers directed from the pSH18-34 reporter suggested that the equilibrium dissociation constants (K_d s) of the interactions were $<10^{-6}$ M. To measure precisely the binding affinity of the aptamers to Cdk2, we used an evanescent wave instrument (BIAcore, Pharmacia). We coupled purified His₆-Cdk2 to CM-dextran chips, then flowed peptide



METHODS. *a*, We purified GST and GST-Cdk2 as described²⁴. We constructed pALHISTRX by annealing the oligonucleotides 5'-TAATGAGCTA-TAAACACCACCACCACCACCAGCAGCAGCACAAAGG-3' and 5'-TACCTTT-GTCGTGTCGTGTCGGTGTCGGTGTCGGTGTTATCGCTCAITTA-3' and ligating into *NdeI*-cut pALTRX-781³. We cloned *AvaII* fragments encoding peptide loops from the library plasmids into *RsrII*-cut pALHISTRX. We expressed His₆-TrxA and His₆ aptamers in G1724 as described²⁵, purified them on a Ni²⁺-NTA-agarose gel according to manufacturer's directions (Qiagen), and dialysed them against 10 mM HEPES, pH 7.4, 50 mM NaCl. We precipitated 1 μg His6-TrxA or His₆ aptamers with Gst or Gst-Cdk2 sepharose beads as described²⁴, and detected the aptamers by western blot with an anti-TrxA rabbit antiserum and ECL reagents (Amersham). *b*, We crosslinked His₆-Cdk2 in 10 mM MES, pH 6.1, 50 mM NaCl to CM5 chips with an amine-coupling kit (Pharmacia). We flowed purified aptamers in running buffer (HEPES 10 mM, pH 7.4, NaCl, 50 mM) onto the chips at 5 μl min⁻¹. We recorded association and dissociation of the His₆-Cdk2-aptamer complexes as variations in resonance angle with time. Association phase starts upon aptamer injection and dissociation phase upon running buffer injection. We fitted portions of association and dissociation curves that excluded the sudden variations in resonance angle caused by transitions between running buffer and aptamer-containing running buffer, which differed slightly in refractive index ('buffer fluxes'). *c*, We coinfectd 2 × 10⁷ Sf9 cells with recombinant baculoviruses expressing haemagglutinin-tagged Cdk2 and His₆-cyclin E as described^{32,26}. We lysed cells 40 h after infection in 500 μl 1× kinase buffer⁹. We used 5 μl 100-fold diluted extract in 30 μl



reactions. We performed 20-min reactions at 25 °C by adding 2.5 μCi [γ - ^{32}P]ATP (3,000 Ci mmol $^{-1}$), 25 μM ATP, 100 ng histone H1 (Sigma) and indicated amounts of His $_6$ -TrxA or His $_6$ aptamers. We ran samples on 15% SDS-PAGE gels and revealed them using autoradiography.

aptamers in running buffer (10 mM HEPES, pH 7.4, 50 mM NaCl) over the chip, allowed them to bind, and rinsed the chip with running buffer without aptamer (Fig. 3b). We determined association and dissociation rate constants by fitting the association and dissociation phases of at least two runs (typically four runs) for each aptamer to exponential functions using a nonlinear least-squares algorithm as described⁸. We calculated K_d s by dividing dissociation rate constants by association rate constants. Under these conditions all aptamers exhibited K_d s between 30 and 120 nM (Table 1).

Our results indicated that these peptide aptamers might inhibit the activity of Cdk2, perhaps by binding to a face of the molecule and by blocking its interaction with one of its partner proteins or substrates. Accordingly, we tested the ability of the aptamers to inhibit phosphorylation of histone H1 by Cdk2/cyclin E kinase (Fig. 3c). All tested aptamers did so; under standard conditions (10 mM HEPES, pH 7.5, ~2 mM KCl, 0–5 mM NaCl)⁹, apparent half-inhibitory concentrations ranged from 1.5 to 100 nM. To rule out the possibility that a trace bacterial contaminant was responsible for the inhibition, we removed the His₆ aptamer from the Pep2 preparation with a rabbit polyclonal anti-thioredoxin antiserum; this immunodepleted preparation no longer inhibited Cdk2 kinase activity (not shown). Half-inhibitory concentrations of aptamers were lower than the K_d s measured from evanescent wave experiments, consistent with the idea that some of the energy of each interaction is ionic and is reduced by the salt in the evanescent wave instrument running buffer.

In coprecipitation experiments¹⁰, purified Pep2 did not compete with *in vitro* translated cyclin E for binding to *in vitro* translated Cdk2 (not shown). However, inhibition by Pep2 was reversed by addition of a 10-fold excess of histone H1 (not shown), suggesting that at least Pep2 inhibits kinase activity by competing with its H1 substrate.

Previous studies have established that libraries of unconstrained peptides contain sequences capable of recognizing targets *in vitro*^{11–15} and in yeast¹⁶; unlike the sequences reported here, such isolated peptides often bear similarity to natural interactors. By contrast with unconstrained libraries, constrained peptide libraries are less conformationally diverse¹⁷, but this lack of conformational diversity should lower the entropic cost if binding causes the loop to adopt a single conformation¹⁸; we imagine this reduction in entropic cost accounts for the fact that our Cdk2 peptide aptamers recognize their targets with higher affinity than is typically observed for unconstrained peptides^{16,19,20}. This high affinity suggests that peptide aptamers could inhibit protein function *in vivo*, in the simplest case by binding to specific faces of the target molecule and disrupting its interaction with specific partners or effectors.

The ability to generate large numbers of aptamers from combinatorial libraries, taken together with the interaction trap, which offers a powerful selection for those that bind specific proteins, will aid the selection of peptide aptamers against a variety of intracellular targets. The use of such aptamers as inhibitors of protein contacts should aid the dissection of the networks of protein interactions that govern division of higher eukaryotic cells and

greatly ease the analysis of gene function in those metazoan organisms for which isolation of specific missense alleles is now impractical. The analogy with antibodies suggests that peptide aptamers may also be useful in other applications in which immunological reagents are now used, such as ELISAs, immunofluorescence experiments, and sensors, and suggests routes by which their affinity might be increased—for example by increasing their valency, and using existing interaction technology to select mutants that bind more tightly.

Finally, the fact that the variable region is displayed by TrxA, a platform of known structure, should help in the solving of the loop structure by NMR and X-ray difference methods and help guide searches for peptide-mimetic compounds for use as drugs. This first generation of peptide aptamers may lead to recognition modules for intracellular nanotechnologies aimed at destroying, modifying and assembling macromolecules inside cells. □

Received 9 November 1995; accepted 6 February 1996.

- Sherr, C. J. *Cell* **79**, 551–555 (1994).
- Morgan, D. O. *Nature* **374**, 121–134 (1995).
- LaValle, E. E. *et al. Biotechnology* **11**, 187–193 (1993).
- Lu, Z. & Murray, K. S. *Biotechnology* **13**, 366–372 (1995).
- Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. *Cell* **75**, 791–803 (1993).
- Finley, R. L. Jr. & Brent, R. *Proc. natn. Acad. Sci. U.S.A.* **91**, 12980–12984 (1994).
- Estojak, J., Brent, R. & Golemis, E. A. *Molec. cell. Biol.* **15**, 5820–5829 (1995).
- O'Shannessy, D. J., Bringham-Burke, M., Soneson, K. K., Hensley, P. & Brooks, I. *Analyt. Biochem.* **212**, 457–468 (1993).
- Kato, J., Matsushima, H., Hiebert, S. H., Ewen, M. E. & Sherr, C. J. *Genes Dev.* **7**, 331–342 (1993).
- Reymond, A. & Brent, R. *Oncogene* **11**, 1173–1178 (1995).
- Devlin, J. J., Panganiban, L. C. & Devlin, P. E. *Science* **249**, 404–406 (1990).
- Cwiria, S. E., Peters, E. A., Barrett, R. W. & Dower, W. J. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6378–6382 (1990).
- Lam, K. S. *et al. Nature* **354**, 82–84 (1991).
- Songyang, Z. *et al. Curr. Biol.* **4**, 973–982 (1994).
- Scott, J. K. & Craig, L. *Curr. Biology* **5**, 40–48 (1994).
- Yang, M., Wu, Z. & Fields, S. *Nucleic Acids Res.* **23**, 1152–1156 (1995).
- McConnell, S. J., Kendall, M. L., Reilly, T. M. & Hoess, R. H. *Gene* **151**, 115–118 (1994).
- Spolar, R. S. & Record, M. T. Jr. *J. Science* **263**, 777–784 (1994).
- Oldenburg, K. R., Loganathan, D., Goldstein, I. J., Schultz, P. G. & Gallop, M. A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 5393–5397 (1992).
- McLafferty, M. A., Kent, K. A., Ladner, R. C. & Markland, W. *Gene* **128**, 29–36 (1993).
- Ruden, D. M., Ma, J., Li, Y., Wood, K. & Ptashne, M. *Nature* **350**, 250–252 (1991).
- Gietz, D., St Jean, A., Woods, R. A. & Schiestl, R. H. *Nucleic Acids Res.* **20**, 1425 (1992).
- Golemis, E. & Brent, R. *Molec. cell. Biol.* **12**, 3006–3014 (1992).
- Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A. & Moore, D. D. *Nature* **374**, 91–94 (1995).
- Ausubel, F. M. *et al. Current Protocols in Molecular Biology* (Greene and Wiley-Interscience, New York, 1987–1994).
- Desai, D., Gu, Y. & Morgan, D. O. *Molec. Biol. Cell* **3**, 571–582 (1992).

ACKNOWLEDGEMENTS. P.C. and B.C. contributed equally to this work. We thank M. Stahl and C. Bamdad for help with the evanescent wave measurements, F. Ryan, N. Ahmad and R. Finley for reagents and experimental advice, P. Jackson for the GST-Cdk2 construction, L.-H. Tsai for the His₆-Cdk2 plasmid, D. Morgan for providing baculovirus constructs and P. J. Bjorkman, H. C. M. Nelson, P. Kim, L. Buck and members of the Brent laboratory for comments on the manuscript. P.C. was supported by an EMBO long-term fellowship, R.B. was supported by the Pew Scholar's program, Hoechst AG, and an American Cancer Society Faculty Research Award. Starting support for this work came from a small grant for exploration research from the NSF.

CORRESPONDENCE and requests for material should be addressed to R.B. (e-mail://xanadu.mgh.harvard.edu)

Crystal structure of the kinesin motor domain reveals a structural similarity to myosin

F. Jon Kull*, Elena P. Sablin*, Rebecca Lau*, Robert J. Fletterick*† & Ronald D. Vale*†‡

Departments of *Biochemistry/Biophysics and †Pharmacology, and ‡the Howard Hughes Medical Institute, University of California, San Francisco, California 94143, USA

KINESIN is the founding member of a superfamily of microtubule-based motor proteins that perform force-generating tasks such as organelle transport and chromosome segregation^{1,2}. It has two identical ~960-amino-acid chains containing an amino-terminal globular motor domain, a central α -helical region that enables dimer formation through a coiled-coil, and a carboxy-terminal tail domain that binds light chains and possibly an organelle

TABLE 1 Rate and equilibrium binding constants of six aptamers

Aptamer	Dissociation rate constant $\times 10^{-6}$ (s^{-1})	Association rate constant ($M^{-1} s^{-1}$)	K_d (nM)
Pep 2	480 $\pm$ 109	7.474 $\pm$ 270	64 $\pm$ 16
Pep 3	246 $\pm$ 20	2.201 $\pm$ 160	112 $\pm$ 17
Pep 5	428 $\pm$ 16	8.263 $\pm$ 215	52 $\pm$ 3
Pep 8	120 $\pm$ 15	3.122 $\pm$ 23	38 $\pm$ 5
Pep 10	693 $\pm$ 64	6.555 $\pm$ 28	105 $\pm$ 10
Pep 11	484 $\pm$ 25	5.590 $\pm$ 168	87 $\pm$ 7

We fitted association and dissociation curves to exponential functions using a nonlinear least-squares method⁸ and the data analysis program IGOR (Wavemetrics Inc., Lake Oswego, Oregon).

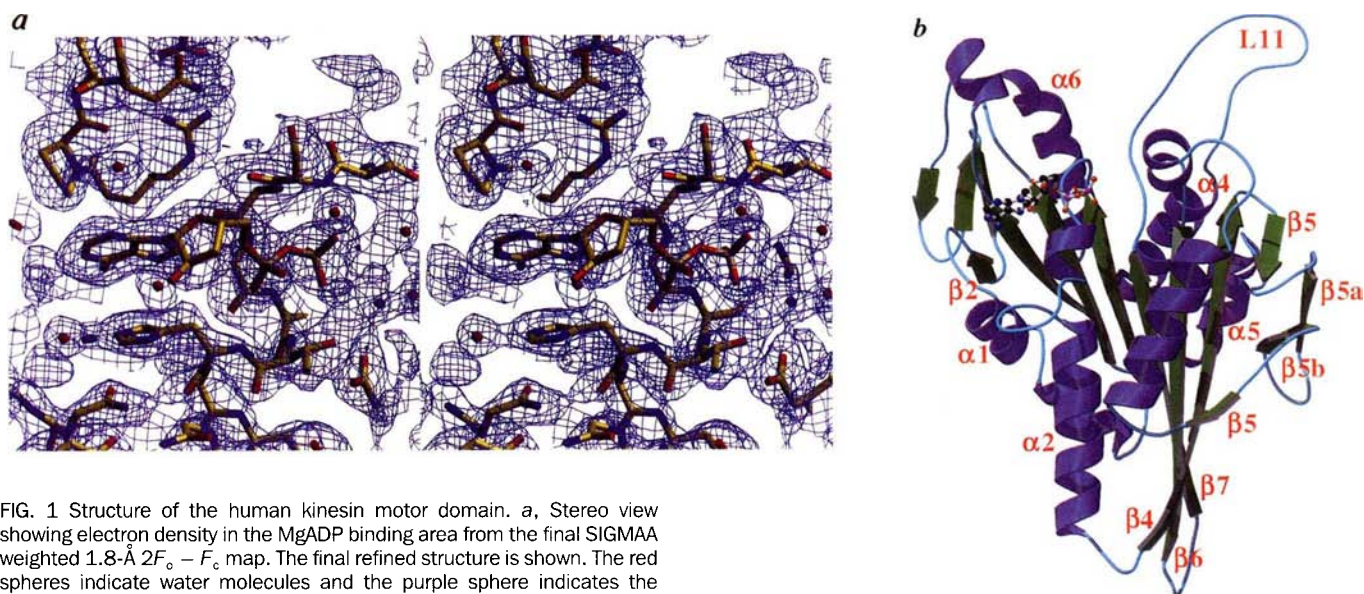


FIG. 1 Structure of the human kinesin motor domain. *a*, Stereo view showing electron density in the MgADP binding area from the final SIGMAA weighted $1.8\text{-}\text{\AA}$ $2F_o - F_c$ map. The final refined structure is shown. The red spheres indicate water molecules and the purple sphere indicates the magnesium ion. Map contours are at 1σ . *b*, MOLSCRIPT³² rendition of the kinesin structure, in which β -strands are shown in green, α -helices in purple, and loops in cyan. The ADP is shown as a ball-and-stick figure. The small lobe containing a three-stranded β -sheet is seen in the upper left of the molecule. Two adjacent strands of the core β -sheet, β_4 (amino acids 126–138) and β_6 (206–216), are unusually long and run the entire length of the molecule; one of the edge strands (β_5 ; 141–144 and 171–173) contains an insertion of 24 amino acids that forms an extended loop with two short β -strands that are isolated from the main β -sheet. Several of the α -helices also exhibit unusual features. Most unusual is α_2 (91–122),

which contains a 9-amino-acid insertion (97–104) flanked by two conserved glycines. This 'hairpin' loop extends perpendicular to the helix, but leaves the overall helical path of α_2 unperturbed. Helices α_4 (257–269) and α_5 (281–292) are notable in that they are oriented at 45° to the β -sheet, unlike the other four helices that are in a parallel orientation. These helices are the only ones linked directly by a short loop rather than by an intervening β -strand. Although loop L11 is shown, it is disordered at the tip (238–254) and is not part of the present model.

receptor¹. The kinesin motor domain of ~340 amino acids, which can produce movement *in vitro*³, is much smaller than that of myosin (~850 amino acids) and dynein (1,000 amino acids), and is the smallest known molecular motor. Here, we report the crystal structure of the human kinesin motor domain with bound ADP determined to 1.8- $\text{\AA}$ resolution by X-ray crystallography. The motor consists primarily of a single α/β arrowhead-shaped domain with dimensions of $70 \times 45 \times 45 \text{\AA}$. Unexpectedly, it has a striking structural similarity to the core of the catalytic domain of the actin-based motor myosin. Although kinesin and myosin have virtually no amino-acid sequence identity, and exhibit distinct enzymatic^{4–6} and motile^{7–10} properties, our results suggest that these two classes of mechanochemical enzymes evolved from a common ancestor and share a similar force-generating strategy.

The structure of the kinesin motor domain (amino acids 1–349) complexed with MgADP was determined by X-ray crystallography using multiple isomorphous derivatives (Table 1; Fig. 1*a*). The fragment of the kinesin motor visible in our structure (amino acids 7–325) is composed of a core eight-stranded, mostly parallel β -sheet that is flanked on each side by three α -helices and a small lobe composed of a three-stranded β -sheet (see Fig. 1 legend for structure description). The MgADP is bound in a rather open and solvent-exposed cleft located in the upper notch of the arrowhead (Fig. 1*b*).

Although kinesin contains a phosphate-binding loop (P-loop) (amino acids 84–92) that is identical in structure to the P-loops of adenylate kinase, transducin, Ras and RecA¹¹, other secondary structural elements of kinesin superimpose poorly with these proteins. In contrast, secondary structural elements of kinesin and myosin were discovered to overlap with one another in a striking manner in the area of the catalytic domain surrounding the nucleotide (Fig. 2*a,b*). Remarkably, seven of the eight core β -strands (the interrupted edge strand, β_5 , being the exception) and all six major helices of the kinesin motor domain overlap with corresponding structural elements in myosin. The r.m.s. deviation

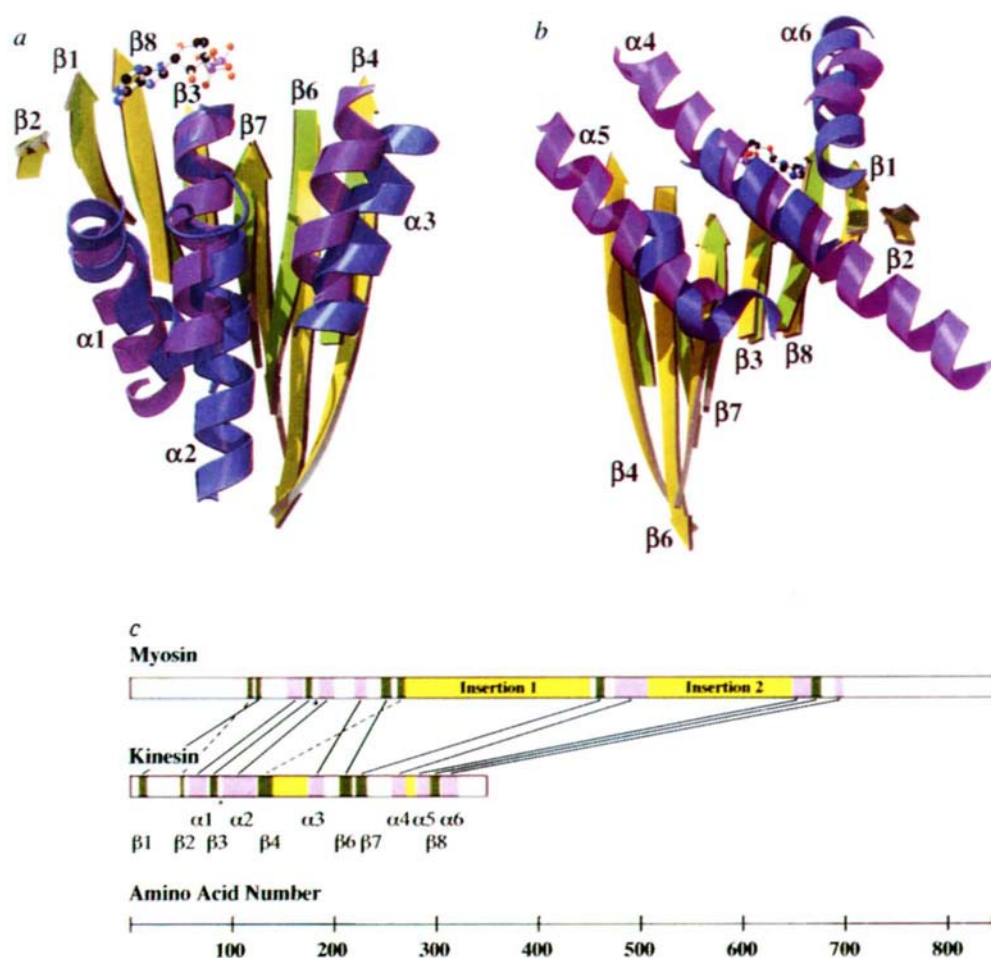
of 183 amino-acid α -carbon positions in the overlapping regions is 3.5 $\text{\AA}$. This result was quite unexpected, given the much greater size of the myosin motor domain and the lack of sequence similarity between the two proteins. A computer alignment of tertiary structures¹² that detected the significant similarity between kinesins and myosins did not detect a similarity between kinesin and any other known protein structure (L. Holm, personal communication).

The structural similarity between kinesin and myosin is not composed of contiguous sequence, being interrupted by large insertions of amino acids (Fig. 2*c*). Of the core structural elements, six of the seven β -strands (the edge strand β_2 being the exception) and all six α -helices run in the same direction. Although the position and direction of these strands and helices are similar, two secondary structural elements are in a different relative location in the primary sequence. These different topological connections can be explained by a few distinct genetic insertions to a common ancestor of the kinesin and myosin families (F.J.K. *et al.*, manuscript in preparation). As well as having longer N- and C-terminal extensions from the core, myosin has two larger insertions compared to kinesin: residues 270–450 (insertion 1), which correspond to a smaller insertion in kinesin (amino acids 138–173); and residues 506–649 (insertion 2), which correspond to loop L12 in kinesin (amino acids 272–280). (Unless indicated, residue numbers refer to chicken skeletal muscle myosin.)

To produce movement, a motor must hydrolyse nucleotide and harness stored chemical energy to generate force while bound to a polymer. The unexpected structural similarity of kinesins and myosins provides an opportunity to identify common features in the mechanisms of motor function. As described below, comparison of the nucleotide-binding region of these two motors shows how conformational changes in this environment can be transmitted and amplified into mechanical work.

The nucleotide-binding cleft is much more open in kinesin than in myosin, even though the rate constant of ADP dissociation from

FIG. 2 Comparison of overlapping secondary structure in kinesin and myosin. The overlap was achieved by aligning nine α -carbon atoms (kinesin amino acids 84–92 and myosin 178–186) in the P-loops of the two structures. The similarity between kinesins and myosins was first noted when the ncd tertiary structure¹⁸ was aligned with other proteins in the coordinate data bank¹² (L. Holm, personal communication). **a**, Front view of the molecule, showing overlapping β -sheets and the three front α -helices. Secondary structure elements of kinesin are indicated. Myosin residue numbers refer to chicken skeletal muscle myosin. Shown are: β -strands (kinesin (k) yellow; myosin (m), green); left to right, β_2 , k50–k52, m116–119; β_1 , k9–k15, m122–m126; β_8 , k295–k302, m668–m675; β_3 , k79–k84, m173–m177; β_7 , k222–k231, m457–m463; β_6 , k206–k216, m247–m255; β_4 , k126–k138, m263–m268. Helices (kinesin, blue; myosin, purple); left to right, α_1 , k58–k74, m155–m169; α_2 , k91–k122, m186–m199; α_3 , k176–k189, m220–m231. **b**, Rear view of the molecule, showing overlapping β -sheets and the three rear α -helices. β -strands, same as in **a**, but rotated by 180°; α -helices, left to right, α_5 , k281–k292, m649–m665; α_4 , k257–k269, m475–m506; α_6 , k306–k320, m690–m697. **c**, Placement of corresponding structural elements in the linear sequence of kinesin and myosin. Helices and strands are indicated in purple and green, respectively. Insertions in the core motor domains are shown in yellow. In myosin, these insertions contain elements that interact with actin³³. Lines connect overlapping structural



elements; broken lines indicate structural elements that are in a different relative location in the primary sequence; the asterisk indicates the position of the P-loops.

kinesin ($\sim 0.005 \text{ s}^{-1}$)^{4,6} is much slower than from myosin (1 s^{-1})¹³. In the kinesin pocket, the adenine is sandwiched by a stacking interaction with His 93 from below and by hydrophobic interactions with Pro 17 and the methylene group of Arg 16 from above (Fig. 3a). The last two residues are positioned similarly to Asn 127 and Pro 128 in myosin. The adenine also binds to water molecules coordinated to Pro 17 and Thr 94. The ribose makes no direct protein contacts. Below the adenine, kinesin features a prominent loop (L5; amino acids 96–106) that disrupts the P-loop helix (α_2), which does not have a counterpart in myosin. Although this loop is found in all members of the kinesin superfamily, L5 does not contain a hydrophobic core and has no direct contact with the nucleotide.

Kinesin lacks certain loops that are present in myosin's nucleotide-binding cleft (Fig. 3a). Two unique myosin loops between amino acids 320–327 and 127–136 cover the nucleotide-binding region and position Ser 324 and Trp 131 within crosslinking distance of the adenine¹⁴. A third unique loop (amino acids 233–246, with Arg 239 at its apex), together with the P-loop, creates a narrow tunnel between the ribose and the γ -phosphate region¹⁵. The corresponding loop (L9; amino acids 190–204) in the kinesin structure contains fewer residues and is positioned further away from the α , β phosphates (Fig. 3a,b). The different loop positions in the two motors may explain why phosphate release following hydrolysis is slow (0.1 s^{-1}) and rate limiting in the myosin ATPase cycle¹⁶, but occurs at much faster rates ($> 13 \text{ s}^{-1}$) in the case of kinesin^{5,6}.

Although several loops near the entrance of the nucleotide

pocket are different in kinesin and myosin, comparison of the γ -phosphate environment of the nucleotide pockets reveals intriguing similarities (Fig. 3b). Both kinesin (amino acids 201–203) and myosin (243–245) contain the universally conserved motif of Ser-Ser-Arg, which occupies a similar position in space. Cross-linking studies with ADP-vanadate¹⁷ and the atomic structure of *Dictyostelium* myosin with bound MgADP-AlF₄ (ref. 15), which is thought to be a MgADP-Pi analogue, indicate that the second member of the triplet, Ser 243, can interact with the γ -phosphate. By analogy, Ser 202 in kinesin, which is only 7 Å away from the β -phosphate, may also interact with the γ -phosphate when ATP is bound in the active site. A second region of similarity is seen in the loop extending from the adjacent β -strand (β_7), where the conserved kinesin sequence Asp-Leu-Ala-Gly-Ser-Glu (amino acids 231–236) covers the rear of the nucleotide cleft in a similar position to a nearly identical sequence in myosin (Asp-Ile-Ala-Gly-Phe-Glu; 463–468) (Fig. 3b). The amide group of Gly 466 in this segment of myosin moves to form a hydrogen bond with the AlF₄-ADP complex, which results in the formation of a new salt bridge between Glu 468 and Arg 245 (ref. 15). These rearrangements have been implicated in transmitting conformational changes from the nucleotide cleft of the actin-binding site¹⁵. Because of its similar location, Gly 234 in kinesin may serve a similar function to Gly 466 in myosin (see also ref. 18). Moreover, we speculate that movement of this glycine towards the nucleotide in the ATP state could bring loops L9 and L11 together, thereby disrupting the Arg 203–Glu 199 charge pair and allowing Glu 236 and Arg 203 to form a salt bridge, as described for myosin.

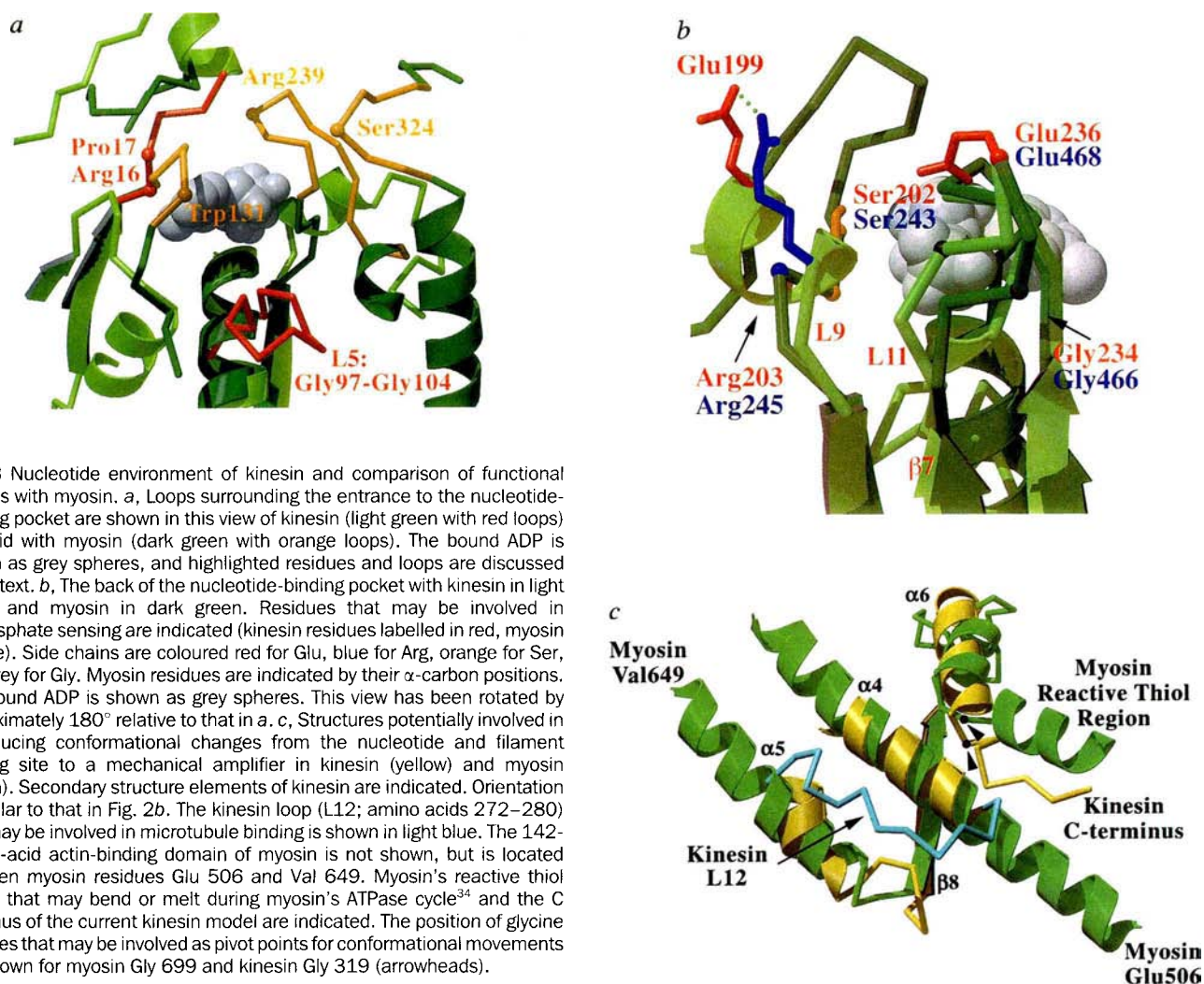


FIG. 3 Nucleotide environment of kinesin and comparison of functional regions with myosin. *a*, Loops surrounding the entrance to the nucleotide-binding pocket are shown in this view of kinesin (light green with red loops) overlaid with myosin (dark green with orange loops). The bound ADP is shown as grey spheres, and highlighted residues and loops are discussed in the text. *b*, The back of the nucleotide-binding pocket with kinesin in light green and myosin in dark green. Residues that may be involved in γ -phosphate sensing are indicated (kinesin residues labelled in red, myosin in blue). Side chains are coloured red for Glu, blue for Arg, orange for Ser, and grey for Gly. Myosin residues are indicated by their α -carbon positions. The bound ADP is shown as grey spheres. This view has been rotated by approximately 180° relative to that in *a*. *c*, Structures potentially involved in transducing conformational changes from the nucleotide and filament binding site to a mechanical amplifier in kinesin (yellow) and myosin (green). Secondary structure elements of kinesin are indicated. Orientation is similar to that in Fig. 2*b*. The kinesin loop (L12; amino acids 272–280) that may be involved in microtubule binding is shown in light blue. The 142-amino-acid actin-binding domain of myosin is not shown, but is located between myosin residues Glu 506 and Val 649. Myosin's reactive thiol region that may bend or melt during myosin's ATPase cycle³⁴ and the C terminus of the current kinesin model are indicated. The position of glycine residues that may be involved as pivot points for conformational movements are shown for myosin Gly 699 and kinesin Gly 319 (arrowheads).

Although the residues of kinesin that interact with microtubules have not been identified by biochemical studies, comparison of the kinesin structure with myosin suggests a possible location for a polymer binding site. The actin-binding interface of myosin is distributed primarily over three regions: a loop from amino acids 405–415, which is part of insertion 1 (Fig. 2*c*), as well as a helical segment (529–558) and a disordered loop (626–647) that belong to insertion 2. The kinesin analogue of myosin insertion 1 is a smaller but well-ordered structure that extends from β 4, and includes the β 5a and β 5b strands and loop L8 (amino acids 138–173). The kinesin analogue of myosin insertion 2 is a short, extended loop (L12, amino acids 272–280) that contains highly conserved hydrophobic residues (Fig. 3*c*). Because these kinesin elements extend from the motor core in the same locations as the actin-binding insertions in myosins, they represent candidate regions for mediating interactions with microtubules.

How might the kinesin motor amplify small sub-nanometre conformational changes in the nucleotide pocket into larger translational movements? The side of the kinesin motor opposite the nucleotide pocket forms a network of structural elements that could transduce conformational changes to the putative microtubule-binding region and to the C terminus of the motor domain, which is connected to the long stalk (Fig. 3*c*). This network includes the putative microtubule-binding site described above (L12; amino acids 272–280), the side chains of which form interactions with both α 4 and α 5. Conformational changes in the filament-binding site could also affect α 6, which is the C-terminal helix visible in the structure. A similar network is seen in

myosin, where two helices corresponding to α 4 and α 5 in kinesin flank the large actin-binding region insert (amino acids 507–648). The myosin equivalent of α 4 is thought to be particularly important in the conformational change mechanism, as it contains highly conserved residues and undergoes rotational movements in different nucleotide states¹⁵. After the actin-binding insertion, myosin then follows a similar path to kinesin through a core β -strand (amino acids 668–675; equivalent to β 8 in kinesin) and into a helix (690–697; equivalent to α 6 helix). The C-terminal region of the kinesin structure corresponds to an area of considerable interest in myosin (the reactive thiol region) that is thought to propagate and amplify nucleotide-dependent conformational changes¹⁵.

The amplifying structure in myosin is probably an 85-Å α -helix that extends from the catalytic core and is stabilized by two associated light chains. This helix can move by 35 Å or more in different nucleotide states^{19,20}. This 'lever arm' action is thought to constitute the myosin power stroke that underlies force generation. Although there is no comparable element visible in kinesin, circular dichroism spectral analysis of bacterially expressed kinesin and synthetic peptides suggest that residues within amino acids 330–370 (the neck region) form a long helix that forms a coiled-coil (R. Hodges, personal communication; T. Shimizu and H. Morii, personal communication). If this helix were stable enough to form a rigid 'lever arm', and undergoes a $\sim 30^\circ$ rotation as observed in myosin²⁰, the translation produced would be ~ 25 Å, much shorter than the 80 Å necessary to move from one tubulin binding site to the next during each ATPase

TABLE 1 Summary of data collection, phase calculation, model building and refinement

	Native	I-ATP	EMTS
Cell dimensions:			
a (Å)	48.54	48.66	48.68
b (Å)	67.94	67.88	67.73
c (Å)	112.95	113.37	112.76
Data collection:			
Resolution (Å)	1.8	2.5*	2.5
R _{sym} (%)†	6.1	17.2	14.1
Observed reflections	101,090	40,935	96,338
Unique reflections	30,582	11,951	12,726
Completeness (%)	82.4	87.4	91.1
Phasing (15.0–3.0 Å):			
R _{scale} ‡	–	14.2	21.2
Number of sites	–	1	3
Relative occupancy	–	–	1.0
			0.63
			0.40
Phasing power§		0.84	1.95
R _{Cullis}		0.76	0.59
Mean figure of merit	0.48		
Refinement (6.0–1.8 Å):			
R _{cryst} (%)¶	23.4		
R _{free} (%)#	30.7		
Average B-factor (Å ²)	26.1		

The first 349 amino acids of the human kinesin gene²⁵ containing the conserved motor domain were expressed in bacteria and purified as described²⁶. Crystals were obtained using sitting drops containing 20 µl of ~5 mg ml⁻¹ protein in 50 mM Na Acetate, pH 4.6, 75 mM KCl, 3.5% (w/v) PEG 4000, 2.5 mM ATP, 10 mM MgCl₂. Crystals (1,000 × 150 × 40 µm) appeared after 2–3 days at 4 °C, and were of the orthorhombic space group P2₁2₁2₁ with one molecule with bound MgADP in the asymmetric unit. All data collection was performed at -170 °C using a 30% (v/v) solution of glycerol/precipitant solution as a cryosolvent. The native data were collected at Stanford Synchrotron Laboratory Radiation beamline 7-1 (λ = 1.08 Å), and derivative data were collected using a 300-mA, 50-kV rotating anode X-ray source. The first derivative was obtained by co-crystallizing K349 with 2.5 mM 2'-iodo-ATP (I-ATP)²⁷ instead of ATP. The second derivative was obtained by soaking native crystals in the presence of 0.1 mM ethyl mercurothiosalicylate (EMTS) for ~2 h. The programs DENZO and SCALEPACK (Z. Otwinowski and W. Minor) were used to index and integrate all data sets. Heavy-atom sites were initially found by analysis of difference Patterson maps and refined using the program XTALVIEW²⁸. One I-ATP derivative site and three Hg sites (bound to Cys 174, Cys 168 and Cys 294) were found for each kinesin molecule. Heavy-atom positions were further refined, and multiple isomorphous replacement (MIR) phases were calculated using the program MLPHARE, and the initial 3.0-Å MIR map and density-modified map (solvent flattening/histogram matching using the program DM) were calculated using the CCP4 suite of programs²⁹. The K349 chain could be traced using these maps in combination with density skeletons created using the program O (T. A. Jones and M. Kjeldgaard). The quality of initial maps was better than expected from the phasing statistics, as indicated by visible carbonyl density in several helices. After a chain of ~160 alanines and 40 residues was placed, the chain was compared with the working model of the NCD structure. Comparison of the two independently derived chains confirmed that the chains were correctly placed and connected. An initial, four-fragment model of a ~275-amino-acid fragment of the kinesin motor was then refined in the program X-PLOR³⁰ using a least-squares protocol and, later, least-squares and simulated annealing protocols. Individual B-factors were refined isotropically. The structure satisfied the PROCHECK³¹ criteria at 1.8 Å. The final structure contains amino acids 7–325, MgADP and 30 water molecules.

* I-ATP data were processed to 2.5 Å; diffraction was weak beyond 3.0 Å.

† R_{sym} = 100 × Σ_{hkl} Σ_i |I_i - I| / Σ_{hkl} Σ_i I_i. All positive, non-zero reflections (0 σ intensity cut-off) were included in scaling and merging.

‡ R_{scale} = 100 × Σ_{hkl} |F_{PH} - F_H| / Σ_{hkl} |F_P|. All positive, non-zero reflections were included in this calculation.

§ Phasing power = (F_H) / (E), where (F_H) is the mean calculated heavy-atom structure factor amplitude, and (E) is the mean estimated lack of closure. All positive, non-zero reflections were included in this calculation.

|| R_{Cullis} = (E) / (iso), where (E) is the mean estimated lack of closure and (iso) is the isomorphous difference.

¶ R_{cryst} = 100 × Σ_{hkl} |F_o - F_c| / Σ_{hkl} |F_o|, where F_o and F_c are observed and calculated structure factor amplitudes, respectively (for all data).

R_{free} was calculated using 10% of the data chosen randomly and omitted from the refinement.

cycle^{9,10}. However, movement of this putative amplifier in the native kinesin dimer could communicate mechanical strain to the partner head, biasing it to attach to a forward binding site on the microtubule²¹. This could be accomplished by relatively small conformational changes in the leading motor head that could be communicated to the partner head through a linkage between the two polypeptide chains. This cooperative mechanism could also explain how kinesin moves for long distances along a microtubule without dissociating^{7,8,22,23}, whereas myosin spends most of its ATPase cycle dissociated from actin²⁴.

In conclusion, the considerable structural similarities between kinesin and the core of myosin suggest that these two motor proteins may adopt a similar strategy for converting chemical energy into mechanical work. Kinetic differences in the ATPase cycles may result from interactions of the nucleotide with loops that are unique for each motor. However, our data show that residues and structural elements mediating conformation changes between ATP and ADP states may be similar in the myosin and kinesin superfamilies (see also ref. 18). Moreover, amplification of conformational changes may occur in the C-terminal region just

beyond the core of both motor proteins. Adaptations in this amplifier region may enable different types of motility between kinesin and myosin as well as between motor proteins within a superfamily. □

Received 28 December 1995; accepted 16 February 1996.

- Bloom, G. & Endow, S. *Motor Proteins 1: Kinesin* (Academic, London, 1994).
- Goldstein, L. S. *A. Rev. Genet.* **27**, 319–351 (1993).
- Yang, J. T., Saxton, W. M., Stewart, R. J., Raff, E. C. & Goldstein, L. S. *Science* **249**, 42–47 (1990).
- Hackney, D. D. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6314–6318 (1988).
- Gilbert, S. P., Webb, M. R., Brune, M. & Johnson, K. A. *Nature* **373**, 671–676 (1995).
- Ma, Y. Z. & Taylor, E. W. *Biochemistry* **34**, 13233–13241 (1995).
- Howard, J., Hudspeth, A. J. & Vale, R. D. *Nature* **342**, 154–158 (1989).
- Block, S. M., Goldstein, L. S. & Schnapp, B. J. *Nature* **348**, 348–352 (1990).
- Svoboda, K., Schmidt, C. F., Schnapp, B. J. & Block, S. M. *Nature* **365**, 721–727 (1993).
- Coppin, C. M., Finer, J. T., Spudis, J. A. & Vale, R. D. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Story, R. M. & Steitz, T. A. *Nature* **355**, 374–376 (1992).
- Holm, L. & Sander, C. *J. molec. Biol.* **233**, 123–138 (1993).
- Bagshaw, C. R. & Trentham, D. R. *Biochem. J.* **141**, 331–349 (1974).
- Grammar, J. C., Kuwayama, H. & Yount, R. G. *Biochemistry* **32**, 5752–5732 (1993).
- Fisher, A. J. et al. *Biochemistry* **34**, 8960–8972 (1995).
- Taylor, E. W. in *The Heart and Cardiovascular System* (ed. Fozzard, H. A.) 1281–1293 (Raven, New York, 1992).

17. Yount, R. G., Cremona, C. R., Grammar, J. C. & Kerwin, B. A. *Phil. Trans. R. Soc. Lond. B* **336**, 55–61 (1992).
18. Sablin, E. P., Kull, F. J., Cooke, R., Vale, R. D. & Fletterick, R. J. *Nature* **380**, 555–559 (1996).
19. Whittaker, M. et al. *Nature* **378**, 748–751 (1996).
20. Jontes, J. D., Wilson-Kubalek, E. M. & Milligan, R. A. *Nature* **378**, 751–753 (1995).
21. Peskin, C. S. & Oster, G. *Biophys. J.* **68**, 202s–211s (1995).
22. Vale, R. D. et al. *Nature* **380**, 451–453 (1996).
23. Berliner, E., Young, E. C., Anderson, K., Mahtani, H. K. & Gelles, J. *Nature* **373**, 718–721 (1995).
24. Spudis, J. A. *Nature* **372**, 515–518 (1994).
25. Navone, F. et al. *J. Cell Biol.* **117**, 1263–1275 (1992).
26. Fujiwara, S., Kull, F. J., Sablin, E. P., Stone, D. B. & Mendelson, R. A. *Biophys. J.* **69**, 1563–1568 (1995).
27. Naber, N., Matuska, M., Sablin, E. P., Pate, E. & Cooke, R. *Protein Sci.* **4**, 1824–1831 (1995).
28. McRee, D. E. *Practical Protein Crystallography* (Academic, San Diego, 1993).
29. Collaborative Computing Project No. 4 *Acta crystallogr.* **D50**, 760–763 (1994).
30. Brunger, A. T. *X-PLOR Version 3.1. A system for X-ray Crystallography and NMR* (Yale Univ. Press, New Haven, CT, 1992).
31. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. *J. appl. Crystallogr.* **26**, 283–291 (1993).
32. Kraulis, P. J. *J. appl. Crystallogr.* **24**, 946–950 (1991).
33. Rayment, I. et al. *Science* **261**, 58–65 (1993).
34. Wells, J. A. & Yount, R. G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4966–4970 (1979).

ACKNOWLEDGEMENTS. We thank N. Naber for preparation of 2'-I-ATP, C. Coppin for discussing models of kinesin movement, and P. Focia, P. Foster, L. Brinen, T. Stout, A. McDonald and F. Malik for help. This work was supported by NIH Program Project grants.

CORRESPONDENCE and requests for material should be addressed to R.D.V. Coordinates referred to in this paper will be deposited in the Brookhaven Protein Database within a year of publication.

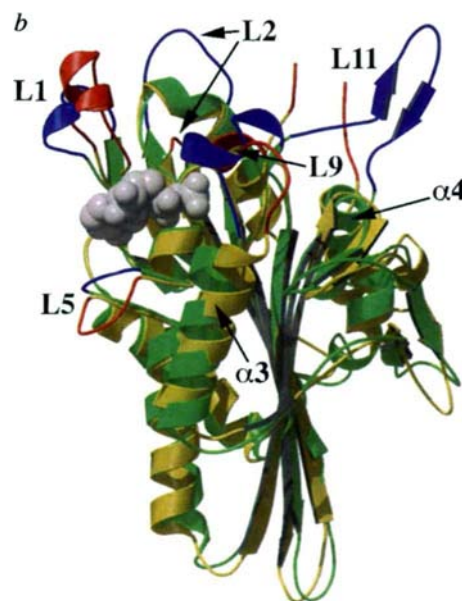
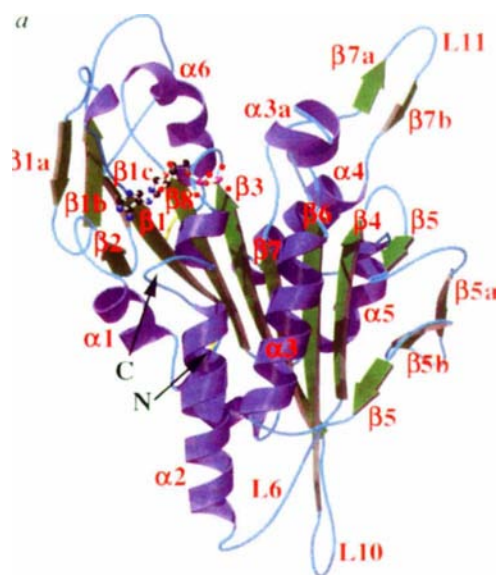
Crystal structure of the motor domain of the kinesin-related motor *ncd*

Elena P. Sablin*, F. Jon Kull*, Roger Cooke*, Ronald D. Vale*†‡ & Robert J. Fletterick*†

Departments of * Biochemistry/Biophysics and † Pharmacology, and ‡ the Howard Hughes Medical Institute, University of California, San Francisco, California 94143, USA

MICROTUBULE-BASED ATPases of the kinesin superfamily^{1,2} provide the motile force for many animated features of living cells. Kinesin motors differ in their direction of movement along microtubules. Kinesin³ and *ncd*^{4,5}, a kinesin-related motor involved in formation and maintenance of mitotic and meiotic spindles, move in opposite directions along microtubules, even though their motor domains are 40% identical in amino-acid sequence. Here we report the crystal structure of the MgADP complex of the *Drosophila ncd* motor domain determined to 2.5 Å by X-ray crystallography, and compare it to the kinesin structure. The *ncd* and kinesin motor domains are remarkably similar in structure, and the locations of conserved surface amino acids suggest these motors share a common microtubule-binding site. Moreover, structural and functional comparisons of *ncd*, kinesin, myosin and G proteins indicate that these NTPases may have a similar strategy of changing conformation between NTP and NDP states. We propose a general model for converting a common γ -phosphate-sensing mechanism into opposite polarities of movement for kinesin and *ncd*.

Motility and directionality of *ncd* and related motor proteins of the kinesin superfamily^{6,7} is determined by a motor domain⁸ located at the carboxy terminus of the molecule. The structure



acids that form an extended loop L8 with two short antiparallel β -strands on its opposite sides. A pair of adjacent strands, $\beta 6$ and $\beta 7$, are unusually long. The tight turn that connects them, the L6 loop and the C-terminal part of the $\alpha 2$ helix form the point of the arrowhead. The latter helix is interrupted by the 'hairpin' loop L5, which is bordered by two conserved glycines Gly 446 and Gly 452 and extends perpendicular to the helix. Helices $\alpha 4$ and $\alpha 5$, which are oriented at 45° to the β -sheet (the other four are in a parallel orientation) and connected directly by a short loop L12, are discussed in the text as being possibly involved in the nucleotide-dependent conformational changes. Helix $\alpha 1$ is bent at Pro 417. Separate from the core in the upper left of the molecule lie three β -strands ($\beta 1a$, $\beta 1b$ and $\beta 1c$) which form a smaller lobe that contacts the nucleotide. *b*, Superposed structures of *ncd* and kinesin motor domains. Areas similar for both motors are shown in yellow for kinesin and green for *ncd*. Variant surface loops are red for kinesin and blue for *ncd*. The ADP of *ncd* is drawn as a space-filling model. The view features three variant loops (L1, L5 and L9) that form a more 'closed' nucleotide-binding pocket in *ncd* compared to kinesin, and highlights the exposed surface loop L11 (part of the loop is missing in the current kinesin structure) and L2, which differs in size between *ncd* and kinesin. The helices $\alpha 3$ and $\alpha 4$ have the largest r.m.s. deviations among the core secondary structure elements between the two motors.

FIG. 1 Structure of the *ncd* motor domain and its comparison to the kinesin motor. *a*, The course of the *ncd* polypeptide chain; α -helices are shown in purple, β -strands in green, and loops in cyan; the nucleotide is drawn as a ball-and-stick model. The assignments of secondary structure elements are indicated. The core of the arrowhead-shaped *ncd* motor domain is composed of an eight-stranded β -sheet, flanked by six α -helices. The $\beta 5$ strand at the right edge of the β -sheet is interrupted by an insertion of 26 amino

of the ncd motor domain (Arg 335–Lys 700)⁹ complexed with MgADP was determined using the multiple isomorphous replacement (MIR) method (see Table 1). The current ncd model includes 321 amino-acid residues (Gly 347–Val 667) and appears as an asymmetric arrowhead with dimensions of $\sim 75 \times 45 \times 45 \text{ \AA}$ (Fig. 1a). The molecule is elegant in its simplicity. The core of the motor is composed of an eight-stranded β -sheet, flanked by three α -helices on each side. Separate from the core lie three antiparallel β -strands that form a small lobe of the motor. The MgADP is bound in an exposed surface cleft (see Fig. 1a legend for structure description).

Although ncd and kinesin move in opposite directions, their three-dimensional structures are remarkably similar (Fig. 1b). The central β -sheet and six α -helices that constitute the core, as well as the three β -strands that form the small lobe, are essentially identical in both motors. Their core structural elements (146 α -carbon atoms) can be superimposed with an r.m.s. deviation of 1.21 $\text{\AA}$. The most noticeable differences among the core residues are in helices α_3 (r.m.s. deviation of 2.32 $\text{\AA}$ for 14 α -carbons) and α_4 (r.m.s. deviation of 2.14 $\text{\AA}$ for 15 α -carbons). The position of the MgADP is virtually identical (r.m.s. deviation of 0.74 $\text{\AA}$) for both motors.

The largest differences between kinesin and ncd are found in their surface loops. Surrounding the entrance to the nucleotide-binding cleft is a group of loops (L1, L5 and L9) that are all directed closer to the nucleotide in ncd than in kinesin. The most unusual of these is the hairpin loop L5 that interrupts helix α_2 and is shorter in the case of ncd. A second area of difference is found in their small lobes. Notably, the loop L2 in ncd contains 10 additional residues (amino acids 385–394; unless noted, all amino-acid residue numbers described in the text are for ncd), and extends further from the main body of the motor compared to kinesin. Based upon sequence alignments, the size of this loop is variable among members of the kinesin superfamily, but is similar among subclasses of kinesin motors. A third distinctive region in the ncd and kinesin motor domains is the extended $\sim 20\text{-\AA}$ surface loop L11 that connects β_7 and α_4 . In ncd, L11 is well ordered and stabilized by two short antiparallel β -strands on opposite sides of the loop, whereas in kinesin this loop is disordered and absent in the current model.

The N- and C-terminal segments of the ncd and the kinesin motor domains would also be

expected to be different, because they are attached to their respective α -helical stalks through opposite ends of their polypeptide chains. Strikingly, the N and C termini are located $\sim 10 \text{ \AA}$ from each other and are positioned similarly in space in the current ncd and kinesin models (Fig. 1a). Unfortunately, the N

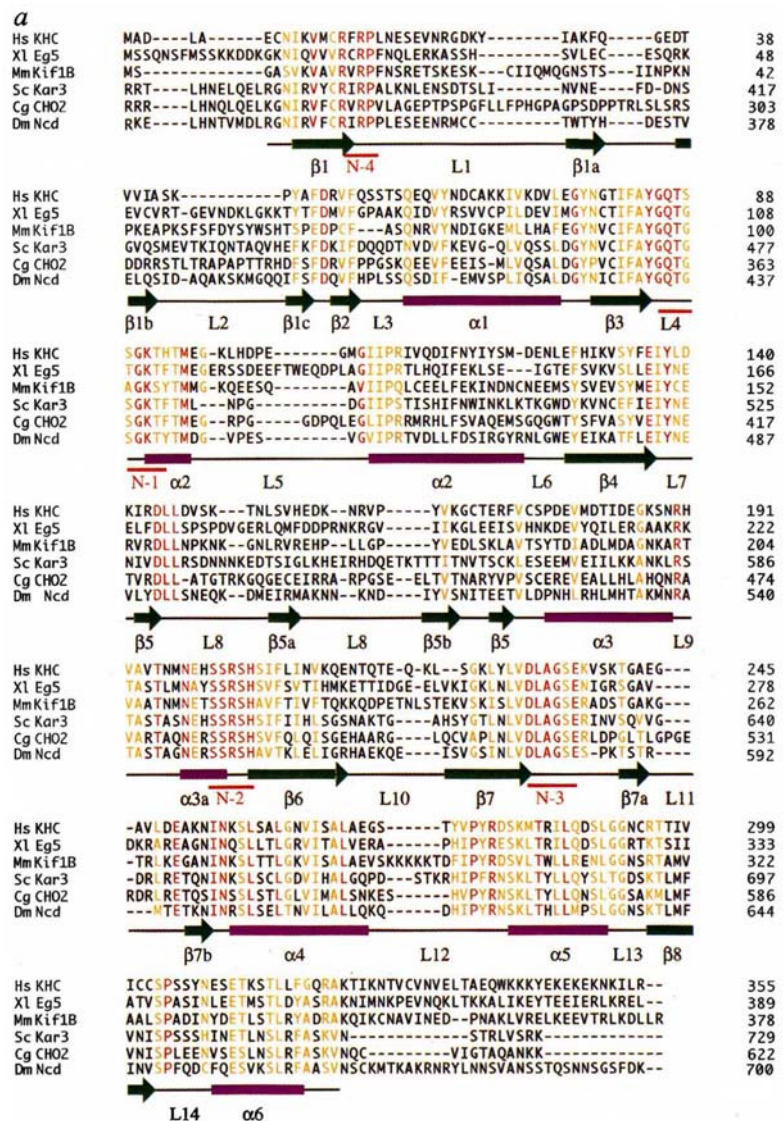


FIG. 2 Sequence alignment of the plus- and minus-end directed motor domains of the kinesin superfamily and location of conserved residues in the ncd structure. a, The top three and the bottom three motors are known to move towards the plus-end and the minus-end of the microtubule, respectively. Sequence comparisons were made between 40 kinesin motor domains from a variety of species using GeneWorks (IntelliGenetics) (alignments of all motors are not shown). Residues that are absolutely conserved (red), or that only have conservative substitutions in 38 of 40 kinesin motor domains (orange), are indicated. The positions of secondary structure elements as well as the location of conserved motifs (N1–4) are indicated. b, Locations of the most highly conserved residues (red) in the ncd structure (same view as Fig. 1a). The locations of conserved motifs (N1–4) are indicated on the left. The right side shows a slab view highlighting the areas that may be involved in γ -phosphate sensing ('switch I' and 'switch II') and microtubule binding (L12).

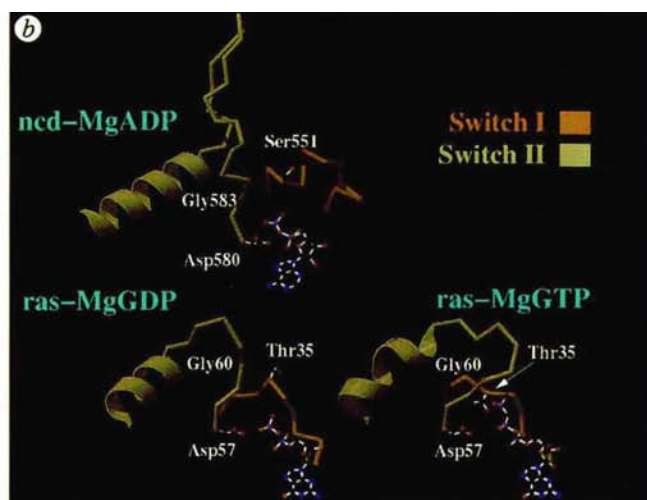
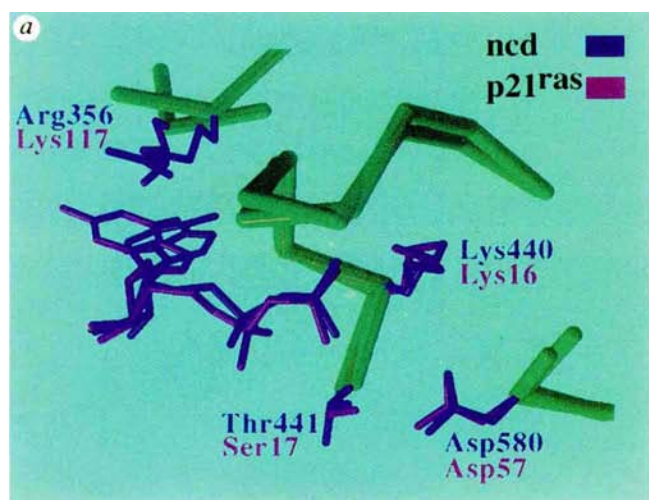


FIG. 3 The nucleotide environments of *ncd* and *p21^{ras}* and the architecture of the switch regions. **a**, Superposition was performed using α -carbon positions of residues from the P-loops (GxxxGKS/T; amino acids 434–441 in *ncd* and 10–17 in *p21^{ras}*). The polypeptide backbones are shown in green. The ribose and the α - and β -phosphates of nucleotides, side chains of the Lys and Ser/Thr from the P-loops (G-1 or N-1), Asp of G-3/N-3, and Lys/Arg of G-4/N-4, are shown in purple for *ncd* and blue for *p21^{ras}*. **b**, The 'switch I' and 'switch II' regions of *p21^{ras}* (ref. 12) in MgGDP and MgGTP (as defined by a non-hydrolysable analogue MgGDP-CP) states and the MgADP state of *ncd* are shown. In the case of *p21^{ras}*, the 'switch' regions that

change conformation upon GDP–GTP exchange are composed of loop L2 ('switch I') and loop L4 and following helix α 2 ('switch II'). The conserved Thr (Thr 35 of *p21^{ras}*) in 'switch I' and the invariant Gly (Gly60 of *p21^{ras}*) in 'switch II' regions form hydrogen bonds with the γ -phosphate of GDP-CP. The proposed 'switch I' region in *ncd* is composed of loop L9, and the 'switch II' region corresponds to the loop L11 and the following helix α 4. Positions of an analogous Ser (Ser 550) and the invariant Gly (Gly583) are indicated. The Asp of G-3/N-3, which is also shown in **a**, are also shown for both *ncd* and *p21^{ras}*.

and C termini are disordered in both structures, but they may be better ordered in other nucleotide states or in the native dimeric motors.

Comparison of electrostatic potential surfaces of *ncd* and kinesin motor domains indicated that they have two distinct sides: the nucleotide-binding face, which is predominantly negative, and the opposite face, which is preferentially positively charged (data not shown). Although the distribution of charge clusters on the nucleotide face is rather different for two motors, the locations of positive charges on the opposite face are identical and correspond to the surface loops L8, L9 and L12.

To gain insight into the possible function of the conserved residues in the kinesin family, their locations in the three-dimensional structure of *ncd* were examined (Fig. 2). Many of the highly conserved residues cluster in regions that are involved in nucleotide binding (Fig. 2b). The two conserved arginines and the proline at the end of β 1 strand (RxRP, amino acids 354–357) interact with the adenine ring. Other conserved residues appear to be important for interacting with the nucleotide phosphates. In addition to the P-loop residues (L4; GQTxSGKT, 434–442), two groups of conserved residues (NxxSSRSH, 539–554; and DLAGxE, 580–585) are found at the ends of adjacent strands β 6 and β 7. These two clusters straddle either side of a plane defined by the phosphate backbone and, as described later, may be involved in the γ -phosphate sensing mechanism.

Close in space to the NxxSSRSH and DLAGxE motifs are two additional clusters of highly conserved residues: ExYxEx/DxxxDLL (483–493; L7 and L8) and I/V-P-F/Y-R (620–623; L12). The latter loop is unusual in that it contains solvent-exposed hydrophobic side chains. The conservation of amino-acid residues, the steric and charge similarities between *ncd* and kinesin in L8, L9 and L12 (see above) and the correspondence of L8 and L12 to actin binding insertions in myosin (see ref. 10) suggest that they may be microtubule-binding sites.

There are also class-specific conserved residues in interesting areas in the *ncd* and kinesin structures. An example is the motif Gly-Trp of *ncd* (amino acids 473 and 474), which is located in loop L6 and is absolutely conserved among C- but not N-terminal kinesin motors. Such areas could be important in establishing directionality.

The most conserved residues in the kinesin superfamily cluster around the nucleotide and the proposed microtubule-binding site, suggesting that they may be part of a signal pathway that controls the binding and release of the motor from the microtubule during transitions in the ATP hydrolysis cycle. The mechanism by which nucleotide hydrolysis can affect protein–protein interactions is best understood for proteins of the GTPase (G-protein) superfamily, as several G-protein structures have been determined with GDP or GTP analogues bound in the active site^{11–16}. Like motor proteins, G proteins undergo a nucleotide hydrolysis cycle in which the exchange of GDP for GTP is coupled to conformational changes that alter their ability to bind other macromolecules^{17,18}. To investigate whether kinesin motors and G proteins share structural as well as functional similarities, the three-dimensional structure of *ncd* was superimposed by aligning their phosphate-binding loops (P-loops) with MgGDP complexes of G proteins representative of different classes: *p21^{ras}* (ref. 11), EF-Tu (ref. 13) and transducin- α (ref. 15).

In contrast to the structural similarities between kinesins and myosins noted in ref. 10, most secondary structural elements of kinesins and G proteins do not align closely in space. Remarkably, however, the ribose, and the α , β -phosphates of their bound nucleotides are virtually superimposable (Fig. 3a). Moreover, four absolutely conserved sequence motifs (including the P-loop) in the kinesin and G-protein families are located in equivalent positions in space. Similarities in the same motifs were also noted between kinesin and myosin motors (see ref. 10). In G proteins, these regions interact with nucleotide and are termed G-1 (the P-loop), G-2 (D-(x)_n-T), G-3 (DxxG) and G-4 (N/TKxD)¹⁸. The G-2 region is located in a position equivalent to the N-2 region of motor proteins (NxxSSR, amino acids 547–552; Fig. 2). In particular, the G-2 threonine is equivalent to a N-2 serine (Ser 550 or Ser 551). The G-3 region corresponds to N-3 in motors (DxxGxE, 580–585; Fig. 2). Notably, the side chains of the Asp (Fig. 3a) and the α -carbon of the glycine residues are found in virtually identical locations in G proteins and *ncd*. The G-4 region is also located in a similar position to N-4 in motor proteins (RxRP for kinesins (354–357; Fig. 2) and NP for myosins (chicken skeletal myosin, 127 and 128)). The lysine of G-4 and the second arginine of kinesin's N-4 superimpose and both form

contacts with the purine ring of the nucleotide (Fig. 3a).

As demonstrated for several members of the G-protein superfamily, the conserved G-2 and G-3 motifs are parts of the 'switch' regions that are involved in γ -phosphate sensing during the GTP-hydrolysis cycle, and undergo major conformational changes between GTP and GDP states^{12,18}. The 'switch' mechanism of G proteins relies upon two residues, a threonine in 'switch I' and a glycine in 'switch II' that converge on the nucleotide to form a hydrogen bond with the γ -phosphate in the GTP state (Fig. 3b)¹². In kinesins and myosins, the presence of similar key amino acids in locations equivalent to the G-protein 'switch' regions suggest that N-2 and N-3 motifs may function in a similar manner in motor proteins as well. In kinesins, a conserved serine (Ser550 or possibly Ser551) may be equivalent to the 'switch I' threonine, and the invariant glycine (Gly583) may play the role of the G-protein 'switch II' glycine (Fig. 3b). The idea that motors and G proteins may have a comparable γ -phosphate sensing mechanism is supported by findings that the 'switch I' serine and 'switch II' glycine analogues in myosin (Ser243 and Gly466 in chicken myosin) move several ångströms to form hydrogen bonds to the fluorine atoms in the MgADP-AlF₄ (a MgADP-Pi analogue) structure¹⁹.

The trigger movements of the residues that form hydrogen bonds to the γ -phosphate must then be amplified to change their interactions with other macromolecules: biopolymers for motors, and receptors/effectors for G proteins. In the case of G proteins, amplification is accomplished by a rearrangement of the 'switch II' loop and helix (Fig. 3b), which creates a new binding interface for target proteins^{12,18,20,21}. It is striking that motor proteins have comparable structural counterparts (L11 and $\alpha 4$ in ncd (Fig. 3b) and amino acids 463–506 in myosin) to the 'switch II' loop and helix in G proteins. The 'switch II' loop, however, is noticeably longer in kinesin motors (Fig. 3b).

Several observations suggest that movements in loop L11 and helix $\alpha 4$ may occur between ADP and ATP states in kinesin motors, as they do in G proteins. In addition to their similar position to the 'switch II' region of G proteins, helix $\alpha 4$ is poorly packed and makes fewer contacts than other helices. This unusual structural feature may reflect the ability of this helix to change its conformation. Movements in the $\alpha 4$ helix probably affect the $\alpha 5$ helix and the intervening loop L12, as they are interlocked in the MgADP state. Because L12 may contact the microtubule, nucleotide-induced reorientation of L11 and helix $\alpha 4$ may provide a mechanism for communicating between distant nucleotide- and microtubule-binding sites. A similar strategy may also occur in myosins, as elements

comparable to kinesin L11 and $\alpha 4$ move and close a large cleft which is thought to change the actin-binding interface¹⁹.

The structures of kinesin and ncd motor domains are similar, and the locations of functionally important residues are identical, so it is likely that kinesin and ncd undergo a similar initial conformational change in response to the presence of absence of γ -phosphate in the nucleotide. Moreover, the kinesin and ncd structures suggest that these two motors share a similar tubulin binding site and are probably oriented in the same direction along the microtubule (see also refs 22–24). How could a similar conformational change give rise to opposite directionality?

One possibility is that 'plus'- and 'minus'-directed motors bind microtubules in a similar manner, but develop different power strokes. If similar nucleotide-induced conformational changes in the 'switch' regions were propagated differently in kinesin and ncd, the partner head could be biased to bind to tubulin subunits in opposite directions on the microtubule (see ref. 10). Candidate regions that could respond differently to a similar 'switch' mechanism in ncd and kinesin are their variant loops and/or

TABLE 1 Summary of data collection, phase calculation, model building and refinement

Space group: <i>I</i> 222						
<i>a</i> = 127.1 Å, <i>b</i> = 122.3 Å, <i>c</i> = 68.0 Å						
Number of ncd molecules per asymmetric unit: 1						
	Native	EMTS K ₂ Pt(NO ₂) ₇	2'-IATP	2-IATP	5-ICTP	
Data collection						
Resolution (Å)	2.5	3.5	3.3	2.5	2.5	2.5
<i>R</i> _{sym} (%) [*]	6.0	10.5	9.6	5.7	6.2	5.7
Unique reflections	16,519	6,115	6,732	16,042	16,708	15,480
Observed reflections	79,056	25,683	26,928	60,959	67,832	58,784
Completeness (%)	85.4	81.7	82.1	83.0	86.1	80.3
Phasing (15.0–2.5 Å)						
<i>R</i> _{scale} (%) [†]	–	22.4	28.7	14.8	14.2	12.8
Sites	–	1	2	1	1	1
Relative occupancy	–	0.48	1.00	0.78	0.76	0.46
			0.58			
Phasing power [‡]	–	1.12	1.56	2.78	1.97	1.17
<i>R</i> _{Cullis} [§]	–	0.61	0.58	0.48	0.56	0.63
Overall figure of merit	0.61					
Refinement (6.0–2.5 Å)						
<i>R</i> _{cryst} (%)	22.4					
<i>R</i> _{free} (%) [¶]	31.8					
r.m.s. bond length, (Å)	0.012					
r.m.s. bond angle (deg)	2.17					
Average B-factor (Å ²)	29.7					

Bacterially expressed ncd motor domain (Arg335–Lys700) (ref. 9) was crystallized as described previously²⁵. X-ray diffraction data for the native ncd crystals and five isomorphous derivatives were measured at –170 °C using an R-AXIS IIC image plate detector. The data were integrated using DENZO, and scaled with SCALEPACK (Z. Otwinowski and W. Minor). 2'-I-ATP was prepared as described²⁶. Heavy-atom sites for all derivatives were independently located using difference Patterson maps and cross-calculated using protein phases derived from other heavy-atom derivatives. Positions, occupancies and effective thermal parameters for the heavy atoms were refined using the package PHASES. The MIR phases to 2.5 Å were improved through solvent flattening. Model building into the experimental electron density map at 2.5 Å was accomplished using the program O (T. A. Jones and M. Kjeldgaard). Later stages of model building included conjugate gradient minimization and refinement by simulated annealing using X-PLOR²⁷. The entire structure was checked using annealed omit maps. As defined in PROCHECK²⁸, 100% of the main-chain atoms are within the allowed regions of the Ramachandran plot (not shown). The current atomic model includes 321 amino-acid residues along with MgADP and 66 water molecules. Neither the original MIR map nor the final maps based on the refined coordinates contain electron density for first 12 N-terminal and last 33 C-terminal residues.

^{*} *R*_{sym} = 100 × $\sum_i |I_i - \langle I \rangle| / \sum_i I_i$.

[†] *R*_{scale} = 100 × $\sum_i |F_{PH} - F_H| / \sum_i |F_P|$.

[‡] Phasing power = $\langle F_H \rangle / \langle E \rangle$, where $\langle F_H \rangle$ is the mean calculated heavy-atom structure factor amplitude, and $\langle E \rangle$ is the mean estimated lack of closure.

[§] *R*_{Cullis} = $\langle E \rangle / \langle iso \rangle$, where $\langle E \rangle$ is the mean estimated lack of closure, and $\langle iso \rangle$ is the isomorphous difference.

^{||} *R*_{cryst} = 100 × $\sum_i |F_o - F_c| / \sum_i |F_o|$, where *F*_o and *F*_c are observed and calculated structure factor amplitudes, respectively.

[¶] *R*_{free} was calculated using 5% of the data chosen randomly and omitted from the refinement.

regions with class-specific amino-acid conservations. Among the latter, the class-specific neck regions adjacent to the core motor domains (amino acids 335–347 and 324–374 in *ncd* and kinesin, respectively) may be involved in conformational change amplification (see ref. 10). The findings that the N and the C termini are close to one another in space and near the 'switch II' helix are consistent with the idea that the neck regions may be important for directionality. Structural and functional studies of dimeric motor proteins are now in progress to test these ideas. □

Received 28 December 1995; accepted 16 February 1996.

- Endow, S. A. & Titus, M. A. *Rev. Cell Biol.* **8**, 29–66 (1992).
- Goldstein, L. S. *A. Rev. Genet.* **27**, 319–351 (1993).
- Vale, R. D. et al. *Cell* **43**, 623–632 (1985).
- McDonald, H. B., Stewart, R. J. & Goldstein, L. S. *B. Cell* **63**, 1159–1165 (1990).
- Walker, R. A., Salmon, E. D. & Endow, S. A. *Nature* **347**, 780–782 (1990).
- Kuriyama, R. et al. *J. Cell Biol.* **129**, 1049–1059 (1995).
- Endow, S. A. et al. *EMBO J.* **13**, 2708–2713 (1994).
- Stewart, R. J., Thaler, J. P. & Goldstein, L. S. *B. Proc. natn. Acad. Sci. U.S.A.* **90**, 5209–5213 (1993).
- McDonald, H. B. & Goldstein, L. S. *B. Cell* **61**, 991–1000 (1990).
- Kuill, F. J., Sablin, E. P., Lau, R., Fletterick, R. J. & Vale, R. D. *Nature* **380**, 550–555 (1996).
- Tong, L., deVos, A. M., Milburn, M. V. & Kim, S.-H. *J. molec. Biol.* **217**, 503–516 (1991).
- Milburn, M. V. et al. *Science* **247**, 939–945 (1990).
- Kjeldgaard, M. & Nyborg, J. *J. molec. Biol.* **223**, 721–742 (1992).
- Kjeldgaard, M., Nissen, P., Thirup, S. & Nyborg, J. *Structure* **1**, 35–50 (1993).
- Lambright, D. G., Noel, J. P., Hamm, H. E. & Sigler, P. B. *Nature* **369**, 621–628 (1994).
- Noel, J. P., Hamm, H. E. & Sigler, P. B. *Nature* **366**, 654–663 (1993).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **348**, 125–132 (1990).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **349**, 117–127 (1991).
- Fisher, A. J. et al. *Biochemistry* **34**, 8960–8972 (1995).
- Sprinzl, M. *Trends biochem. Sci.* **19**, 245–250 (1994).
- Wittinghofer, A. *Cell* **76**, 201–204 (1994).
- Hirose, K., Lockhart, A., Cross, R. A. & Amos, L. A. *Nature* **236**, 277–279 (1995).
- Hoenger, A. & Milligan, R. A. *Molec. Biol. Cell* **6**, 157a (1995).
- Lockhart, A., Crevel, I.M.-T.C. & Cross, R. A. *J. molec. Biol.* **249**, 763–771 (1995).
- Sablin, E. P. & Fletterick, R. J. *Proteins* **21**, 68–69 (1995).
- Naber, N., Matsuka, M., Sablin, E. P., Pate, E. & Cooke, R. *Protein Sci.* **4**, 1824–1831 (1995).
- Brunger, A. T. in *X-PLOR Version 3.1. A System for X-ray Crystallography and NMR* (Yale Univ. Press, New Haven, CT, 1992).
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. *J. appl. Crystallogr.* **26**, 282–291 (1993).

ACKNOWLEDGEMENTS. We thank M. Matuska and N. Naber for preparation of 2'-I-ATP, V. J. Davisson and M. Deras for providing 2'-I-ATP; R. Wagner and S. Gillmor for assistance in data collection; R. Case for performing the sequence alignments; and D. Pierce for comments on the manuscript. This work was supported by an NIH program project grant. E.P.S. was supported by postdoctoral fellowship from the Leukemia Society of America.

CORRESPONDENCE and requests for material should be addressed to R.J.F. Coordinates referred to in this paper will be deposited in the Brookhaven Protein Database within a year of publication.

CORRECTIONS

Oligosaccharide ligands for NKR-P1 protein activate NK cells and cytotoxicity

Karel Bezouška, Chun-Ting Yuen, Jacqui O'Brien, Robert A. Childs, Wengang Chai, Alexander M. Lawson, Karel Drbal, Anna Fišerová, Miloslav Pospíšil & Ten Feizi

Nature **372**, 150–157 (1994)

WE are re-evaluating the experimental system, originally set up in Prague (by K.B., A.F. and M.P.), used in this work. So far we have not been able to reproduce the effects on natural killing reported for NKR-P1 oligosaccharide ligands. The results of our re-evaluation will be published as soon as they become available.

Correspondence should be addressed to T.F. (e-mail: tfeizi@hgmp.mrc.ac.uk). □

Electrons in artificial atoms

R. C. Ashoori

Nature **379**, 413–419 (1996)

A TYPOGRAPHICAL error in equation (2) caused the $\hbar$ terms to be incorrectly substituted by the Greek letter η . The equation should read

$$E_{n,l} = [\hbar\omega_c/2 + \hbar\sqrt{(\omega_c/2)^2 + \omega_0^2(2n + |l| + 1)}]$$

□

YOURS TO HAVE AND TO HOLD BUT NOT TO COPY

The publication you are reading is protected by copyright law. Photocopying copyright material without permission is no different from stealing a magazine from a newsagent, only it doesn't seem like theft.

If you take photocopies from books, magazines and periodicals at work your employer should be licensed with CLA.

Make sure you are protected by a photocopying licence.



The Copyright Licensing Agency Limited
90 Tottenham Court Road, London W1P 0LP
Telephone: 0171 436 5931 Fax: 0171 436 3986

Guide to authors of contributions to *Nature*

Nature is a weekly international journal covering all the sciences. It is intended for an interdisciplinary readership, so all manuscripts should be written clearly and simply. Contributors should pay particular attention to the fact that English is not the first language of many readers. Space is limited and competition severe, so brevity is highly valued.

Nature publishes the following types of article:

Review articles survey recent developments in a topical area of scientific research. Contributors wishing to submit a review should first send a one-page synopsis to the Reviews Coordinator.

- Reviews inform a broad readership about fields where there are rapid, important advances.
- They focus on one topical aspect of a field rather than providing a comprehensive literature survey.
- They can be controversial but in this event should briefly indicate opposing viewpoints. They should not be focused on the author's own work.
- Language should be simple, novel concepts defined and jargon explained.
- Reviews should not be more than 6,000 words and ideally should be shorter. There should be no more than 100 references and ideally half that number. There is no limit to the number of display items or explanatory boxes (used for clarification of technical points or for background material), but reviews do not generally take more than six pages of *Nature*.
- Review articles are often substantially edited by *Nature's* editors in consultation with the author.

Progress articles review particularly topical and fast-moving fields for a nonspecialist readership. They are similar to Reviews except for the following:

- They do not exceed four pages of *Nature* in length, including display items and references. (One printed page of text is about 1,300 words.)
- They focus on current papers of outstanding interest that are setting new standards in a field.
- Because of their topicality, Progress articles should be written and submitted within a few weeks of *Nature's* editors expressing an interest in a synopsis.
- Authors may discuss their own work, but should make it clear in the text if they are presenting a personal, rather than a consensus, view.
- Titles are brief (generally a single line) and relatively informal.

Articles are reports of original scientific research.

- They report novel conclusions of broad general scientific interest.
- They represent a substantial advance in scientific understanding of an important problem.
- They should not be longer than 3,000 words, have more than six display items (with captions of fewer than 300 words) or have more than 50 references.
- They have a 'heading' of up to 80 words, often rewritten by *Nature's* editors, which advertises the content of the paper in general terms. The heading should not contain numbers, abbreviations or measurements unless central to the message of the paper.
- The introduction and summary should be contained in the first two or three paragraphs of the main text.

Letters to *Nature* are short reports focused on a novel, outstanding finding.

- The implications of the main result are of interest to nonspecialists.
- They should not exceed 2½ pages of *Nature*, equivalent to 1,500 words of text with four small display items that have brief captions.
- They have a bold-text first paragraph of not more than 150 words summarizing the rationale for the study, the main result and conclusion. This paragraph is aimed particularly at nonspecialist readers.
- Letters should have fewer than 30 references and no more than four display items. Captions should not exceed 300 words.
- **Articles and Letters** contain a statement at the end of the text: "Correspondence and requests for materials to xxxx". Database accession numbers should be included after this statement.

Preparation of manuscripts

- All manuscripts should be typed, double-spaced, on one side of the paper only.
- Manuscripts should be accompanied by a brief cover letter from the corresponding author, containing a full postal address, telephone and fax number, and e-mail address.
- Five copies of manuscripts and original figures are needed, together with two copies of the covering letter.
- Five copies of relevant related manuscripts in press or submitted for publication elsewhere should be included, clearly marked as such.
- Unless otherwise instructed by the editor handling a manuscript, when sending revised or resubmitted manuscripts, five copies are required, each accompanied by a copy of the authors' response to ref-

erees' and editors' comments on the earlier version.

- Titles should be brief, pertinent and simple, avoiding active verbs, numerical values, abbreviations and punctuation.
- References are numbered sequentially as they appear in the text, tables and figure legends. Only papers that are published or in press should be given numbers: manuscripts submitted or in preparation should be mentioned in the text with a list of authors.
- Reference lists contain only citations to published papers, and do not contain textual material, grant details or acknowledgements.
- Acknowledgements must be brief and appear after the reference list. *Nature* does not publish grant contribution numbers.
- Figures should not be larger than 22 by 28 cm unless unavoidable. They should be marked with the author's name and, where known, the manuscript reference number. One photocopy of the original figures should be provided. All original figures are returned when *Nature* cannot offer to publish a manuscript, but one copy of the manuscript and the photocopy of the figures are kept in confidential files for three months and then destroyed. Detailed instructions for preparation of figures are available from the production department, and digital files of final versions of the figures may be requested.
- Colour figures are welcome but a contribution towards the cost of reproduction is requested. Inability to pay this charge will not prevent *Nature* publishing figures where colour is essential.
- Figures containing protein/nucleotide sequence information should ideally use the three-letter code for amino acids. One column width can accommodate 20 amino acids or 60 base pairs.
- Figure legends should contain fewer than 300 words. They should consist of a brief description of the figure (title, explanation of the parts, explanation of symbols) followed by a telegraphic account of the methods, if appropriate. Multipart figures are discouraged unless the parts are logically connected.
- Tables do not have a 'methods' section. Symbols and abbreviations in the table should be defined immediately below the table, followed by essential descriptive material, all in double-spaced text. Tables should each be presented on a separate sheet of paper.
- As a condition of publication, authors are required to make materials and methods used freely available to academic researchers for their own use. Supporting datasets must be made available at the time of publication either by deposition in the appropriate public database or by distribution on the Internet, together with the relevant accession numbers or site address. In the case of X-ray crystallographic coordinates only, public access may be delayed for up to one year after publication.
- Manuscripts can be submitted to the Editor at *Nature*, Porter's South, Crinan St, London N1 9XW or at *Nature*, 968 National Press Building, Washington, DC 20045-1938. Proofs should be returned by express mail to London.

Other contributions

Nature publishes informal material in each issue, as follows.

- **Correspondence** contributions are short comments on topical issues, anecdotal material or reactions of readers to nontechnical material published in *Nature* (mainly News, Opinion, Book Review or Commentary articles). All are unsolicited; submissions by e-mail to nature@nature.com are preferred (accents on names and places should be clearly indicated). Contributions should be brief (fewer than 500 words).
- **Commentary** articles are informal, editorial comments on topical issues of public interest that have some bearing on scientific research. They are often controversial. Unsolicited manuscripts are considered, but enquiries can be made to the Commentary Editor by e-mail (nature@nature.com) or fax before formal submission as a one-page synopsis or informal letter.
- **News and Views** editorials inform nonspecialist readers about new scientific advances, either as reported in published papers or in the form of meeting reports. Most are commissioned, but proposals can be made to the News and Views Editor in advance of publication of the paper or of the meeting concerned. Authors are not allowed to discuss their own work or work from elsewhere in their laboratory. Detailed guidelines are available on request.
- **Scientific Correspondence** is for informal discussion of topical scientific issues, including material published in *Nature*. Priority is given to contributions of fewer than 500 words. Detailed guidelines are available on request. All contributions are unsolicited.
- **Book Reviews** are all commissioned by the Book Review editor. Unsolicited contributions are not considered.
- *Nature's* editors will request final copy for Commentary, News and Views, Scientific Correspondence and Book Reviews on diskette or via e-mail.

A more detailed Guide to Authors is available on *Nature's* www site:
<http://www.nature.com>
<http://www.america.nature.com>

Doxorubicin in sterically stabilized liposomes

Danilo D. Lasic

Improved liposome stability and drug retention significantly increase the anticancer activity of encapsulated doxorubicin (Doxil), enhancing the effectiveness of chemotherapy and potentially reducing its toxicity.

DESPITE the rapid development of medicinal and pharmaceutical chemistry in recent decades, chemotherapy still represents a major challenge to the patient. The most potent drugs can be very toxic and often less than one per cent of the injected drug molecules reaches the desired cells, whereas the rest damages healthy cells and tissues.

Drug delivery systems can change pharmacokinetics and biodistribution of the encapsulated agents, which can reduce toxic side effects and increase efficacy of the therapy. Liposomes were considered a drug delivery system of choice for systemic applications due to colloidal size, easily controllable surface and membrane properties, large carrying capacity and biocompatibility. Optimistic expectations, however, did not materialize, mostly because of nonspecific reactivity of liposomes resulting in their instability in blood circulation upon systemic application¹. Typically, intravenously administered liposomes are taken up by the cells of the mononuclear phagocytic system in tens of minutes. In addition, liposomes containing drugs with a low water/octanol partition coefficient can lose the encapsulated cargo even more quickly. Neither mechanical nor electrostatic stabilization, which can stabilize liposomes *in vitro*, could effectively pro-

long their blood circulation times, half-life ($t_{1/2}$). It was proposed that the rapid clearance of liposomes is due to the uptake by the immune system, as well as disintegration resulting from electrostatic, hydrophobic and van der Waals interactions of liposomes with plasma protein. The immune system responds to liposomes immediately after injection, and this opsonization tags them for the subsequent macrophage uptake². Whereas electrostatic and hydrophobic interactions can be minimized by using neutral lipids and mechanically very strong bilayers³, van der Waals attraction is ubiquitous. For liposomes with radius r it can be described at distance h as $U = -A(r/h)$, where A is the Hamaker constant.

A major breakthrough was the realization that steric stabilization can effectively reduce nonspecific interactions within the milieu⁴, which resulted in substantial improvement of $t_{1/2}$ of liposomes coated with inert polymers⁴⁻⁷.

Optimal stabilization, as measured by $t_{1/2}$ in mice, was achieved by sterically stabilized liposomes, which had a bilayer composition (hydrogenated phosphatidyl choline/cholesterol, 55/40) 5 mol per cent polymer-lipid, ²⁰⁰⁰PEG-DSPE (polyethylene glycol-distearoyl phosphatidyl ethanolamine). This lipid composition was chosen to provide complete polymer surface coverage without excessive lateral pressure above the surface, which may induce phase transition. Also, the liposome membrane consists of a mechanically cohesive bilayer (elastic area compressibility modulus $K > 3,000 \text{ dyn cm}^{-1}$ as compared with $K \approx 140 \text{ dyn cm}^{-1}$ for lecithin bilayers)⁸. The distearoyl chains were found to be the optimal anchor to retain the large polar head of the polymer-lipid in the bilayer. The surface coating with inert polymer

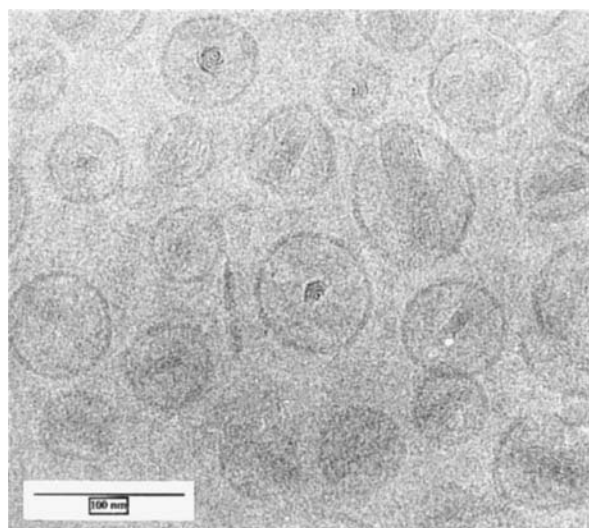


FIG. 2 Cryo-electron micrograph of doxorubicin containing sterically stabilized vesicles. It was shown that the enclosed drug is precipitated in the form of fibres ($[\text{doxorubicin}^+]_2\text{-sulphate}^-$), which are hexagonally packed into bundles with an interfibre separation of 2.8 nm. Detailed theory of loading of doxorubicin into pre-formed liposomes containing ammonium sulphate is described in ref. 28. (Courtesy of P. M. Frederik, Limburg University, Maastricht.)

prevents close approach of macromolecules and reduces van der Waals attraction.

A hypothesis that the prolonged circulation is due to reduced adsorption² of opsonins, was confirmed by osmotic stress and surface force apparatus measurements^{9,10}, which showed substantial steric repulsion up to 5 nm above the bilayer surface (Fig. 1). The presence of nonionic hydrophilic polymer above the bilayer increases repulsive pressure above the membrane. This is due to osmotic and entropic forces originating in the elasticity and limited compressibility of surface-attached polymer chains. All the measured force-distance profiles were in agreement with the scaling description of polymers at interfaces as theoretically described by deGennes¹¹.

The thickness of the coating can be expressed in a good solvent as $h_c = DN(a/D)^{3/5}$ where N is degree of polymerization, a is size of a monomer and D is the mean distance between the grafting points¹¹. Using scaling arguments

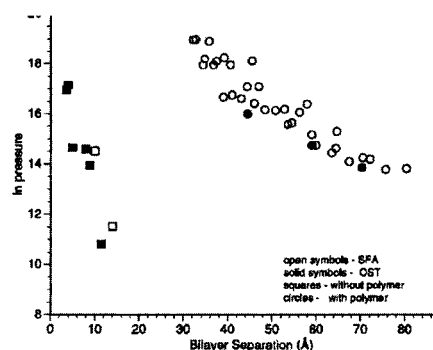


FIG. 1 Force-distance profile of liposomes with (circles) and without (squares) polymer coating as measured by osmotic stress (solid) and surface force apparatus (open) techniques. 4.5 mol per cent of ²⁰⁰⁰PEG-lipid was titrated into liposomes (osmotic stress technique, OST) or into supported bilayers (SFA). (Adapted from refs. 5, 6 and 27.)

(P. Pincus, personal communication), it is possible to estimate polymer thickness for effective stabilization: a general criterion then becomes $A(b/h_c) < kT$, (T is temperature and k is Boltzmann constant). Although scaling theory does not provide numerical prefactor in $h_c/b = A/kT$, one can estimate from the fact that typically, A/kT is of order 1/10, the coating of approximately 5–10 per cent of the particle diameter can give rise to effective steric stabilization.

At 5 mol per cent of polymer lipid with the degree of polymerization around 50 ($\propto D$ and N in the above equation, respectively), the surface is fully coated with polymer chains, which are near

at most hours for conventional liposomes^{4,8,13}. Furthermore, preferential accumulation of these liposomes in tumours or other sites of trauma was observed^{13–15}. This was explained by the fact that blood vasculature pathology at these sites is often leaky and/or badly formed and that particles that can circulate for prolonged times can extravasate into these sites¹. Accumulated liposomes slowly disintegrate and a relatively high concentration of the drug is released at the site¹³. Although the mechanism of release is not yet well understood, it is possible that because liposomes contain excess $(\text{NH}_4)_2\text{SO}_4$ disintegration of the first liposomes releases not only the drug

but also $(\text{NH}_4)_2\text{SO}_4$, which in turn reverses the $(\text{NH}_4)_2\text{SO}_4$ gradient and induces the release of intact liposomes in the vicinity^{1,18}.

In preclinical studies, early treatment in mice has shown complete remission of colon C26 solid tumours¹⁹ (Fig. 3) and mammary carcinoma MC2B 21, and the arrest of growth in human lung cancer xenographs in SCID mice²¹. In these models, free drug, or drug encapsulated in conventional liposomes, were not effective^{1,19–212}. Doxil was also more effective in reducing the incidence of metastases from intramammary implants of tumours MC19 and MC6521.

Following these encouraging results and safety studies, clinical studies were performed^{13–15,22}. To date, more than 2,000 patients with Kaposi sarcoma have been treated with this formulation, which showed reduced toxicity and good response rates¹³. High response rates are due to ten-fold higher drug concentration in tumours ($7.11 \mu\text{g g}^{-1}$ of tissue upon treatment with 40 mg m^{-2}) compared with the administration of free drug (0.72 g g^{-1})¹³. Not unexpectedly, the changed biodistribution of the drug markedly changed its toxicity profile^{14,15,23}. In contrast to myelosuppression as dose-limiting factor, in the case of free doxorubicin, stomatitis seems to be the dose limiting toxicity²². While it was recently observed that conventional doxorubicin-laden liposomes decrease phagocytic activity²³, sterically stabilized liposomes were shown to be less immunotoxic as measured by host resistance to infectious insult²⁴. Based on these data the FDA approved this formulation (Nov. 21, 1995) as the first liposomal therapeutics in the United

States. Currently, it is labelled only as a treatment against Kaposi sarcoma, but recently observed response rates in solid tumours could expand its use.

Several other liposomal anthracycline formulations are in advanced clinical trials (doxorubicin in neutral liposomes by The Liposome Company) or waiting for FDA approval (DaunoXome, liposomal daunorubicin by NeXstar). Both formulations rely on precipitated drug for stable encapsulation and small, neutral vesicles with rigid membrane to improve stability in blood circulation. It seems that, following recent improvements in our understanding of liposome stability and interaction characteristics^{25,26}, liposomes will finally realize their promise as a drug delivery vehicle.

Danilo Lasic is a drug and gene delivery consultant at 7512 Birkdale Drive, Newark, California 94560, USA. For more information fill in reader enquiry number 100 on the reader service card.

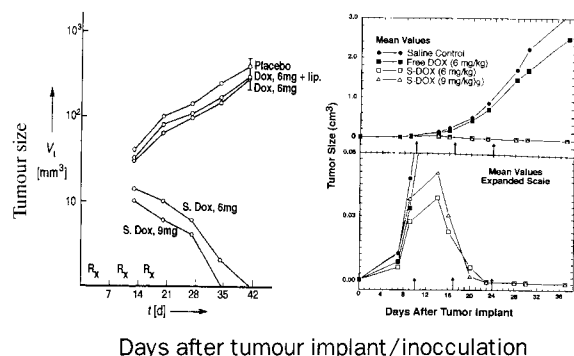


FIG. 3 Antitumour activity of Doxil in a mouse mammary carcinoma MC2B (left) and colon cancer C26 (right) model. Values are days after tumour implantation. The method involved implanting 1.0 mm^3 pieces of mammary carcinoma into mice. In the case of colon carcinoma, 10^6 C26 cells were injected. Animals were treated on day 3, 10 and 17 (left) or 10, 17, 24 (right) with various formulations, and tumour size as a function of time was measured.

mushroom-brush phase transition, where free polymer coils start to overlap and extend^{10,12}.

Doxil are small sterically stabilized unilamellar vesicles ($< 100 \text{ nm}$, composition as above) containing anticancer drug doxorubicin at 2 mg ml^{-1} (ratio total lipid to drug = 7.4 wt wt^{-1}). The drug is practically quantitatively encapsulated due to the fact that it is precipitated in the liposome interior¹⁶ (Fig. 2) and does not leak out¹⁷. In addition to enhanced biological stability of liposomes ($t_{1/2}$) this comprises the second necessary condition for active therapeutics, because most other doxorubicin liposomal formulations are plagued by fast leakage of this relatively hydrophobic drug. Drug is loaded into preformed vesicles by an $(\text{NH}_4)_2\text{SO}_4$ gradient method^{16,17}. The encapsulation is very stable due to the fact that doxorubicin precipitates with sulphate and because there is an excess of $(\text{NH}_4)_2\text{SO}_4$ in the liposomes.

Pharmacokinetic studies of these liposomes have shown $t_{1/2}$ in humans on the order of days, contrasted with minutes or

- Lasic, D. D. *Liposomes: from Physics to Applications*, (Elsevier, Amsterdam, 1993).
- Lasic, D. D., Martin F. & Gabizon, A., Huang, K., Papahadjopoulos, D. *Biochim. biophys. Acta* **1070**, 187–192 (1991).
- Gregoriadis, G. & Senior, J. H. *FEBS Lett.* **119**, 43–47 (1980).
- Papahadjopoulos, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 11640–11644 (1991).
- Klibanov, M., Maruyama, K., Torchilin, V. P. & Huang, L. *FEBS Lett.* **268**, 235–237 (1990).
- Blume, G. & Cevc, G. *Biochim. biophys. Acta* **1029**, 91–97 (1990).
- Senior, J. H., Delgado, C., Fisher, D., Tilcock, C. & Gregoriadis, G. *Biochim. biophys. Acta* **1062**, 77–82 (1991).
- Lasic, D. D. & Needham, D. *Chem. Rev.* **96**, 1601–1624 (1995).
- Needham, D. *et al. J. Liposome Res.* **2**, 411–430 (1992).
- Kuhl, T., Leckband, D., Lasic, D. D. & Israelachvili, J. N., *Biophys. J.* **66**, 1479–1488 (1994).
- deGennes P. G., *Adv. Colloid Interface Sci.* **27**, 189–209 (1987).
- Lasic, D. D. *Angew. Chem. Int. Ed. Eng.* **33**, 1685–1698 (1994).
- Lasic, D. D. & Martin, F. (Eds.) *Stealth Liposomes*, (CRC Press, Boca Raton, 1995).
- Gabizon, A. *et al. Cancer Res.* **54**, 987–992 (1994).
- Gabizon, A. *et al. Acta Oncologica* **33**, 779–786 (1994).
- Lasic, D. D., Frederik, P. M., Stuart, M., Barenholz, Y. & MacIntosh, T. J. *FEBS Lett.* **312**, 255–258 (1992).
- Haran, G., Cohen, R., Bar, L. K. & Barenholz, Y. *Biochim. biophys. Acta* **1151**, 201–215 (1993).
- Lasic, D. D., Ceh, B., Guo, L., Frederik, P. M., Barenholz, Y. *Biochim. biophys. Acta* **1239**, 145–156 (1995).
- Mayhew, E., Lasic, D. D., Babbar, S. & Martin, F. *Int. J. Cancer* **51**, 302–309 (1992).
- Vaage, J., Mayhew, E., Lasic, D. D. & Martin, F. *Int. J. Cancer* **51**, 942–948 (1992).
- Williams, S. S. *et al. Cancer Res.* **53**, 3964–3967 (1993).
- Uziely, B. *et al. J. clin. Oncol.* **13**, 1777–1785 (1995).
- Daemen, T., Hofstede, G., Ten Kate M. T., Bakker-Woudenberg, I. A. J. M. & Scherphof, G. L. *Int. J. Cancer* **61**, 716–721 (1995).
- Zheng, S. *et al. J. Liposome Res.* **3**, 575–558 (1993).
- Lasic, D. D. & Papahadjopoulos, D. *Science* **267**, 1275–1276 (1995).
- Bangham, A. D. *BioEssays* **17**, 1081–1088 (1995).
- Israelachvili, J. N. *Surface and Interparticle Forces*, (Academic Press, New York, 1985).
- Ceh, B. & Lasic, D. D. *Langmuir* **11**, 3356–3368 (1995).

Experimental biology bazaar

Solutions to the complexities of the life sciences are elusive, but what follows may offer a means of saving time as well as energy — several scalable purification systems, an instrument for absolute CD4 counts and more.

CELL ROBOTICS has introduced its new model CellSelector, a **remote-controlled precision microsyringe pump** that is said to handle picolitre volumes and to select and isolate single cells or particles with high purity. The smooth action of this lightweight model is said to provide even greater positioning accuracy and manoeuvrability than before. It allows the user to deliver and withdraw picolitre volumes of fluid in precise, microscopic locations with fingertip control. This design should eliminate flow fluctuations and allow the module to be directly mounted on the microscope stage. The pump's fluid flow is computer controlled when used with Cell Robotics' microscope workstation, allowing continuous or pulsed delivery of minute volumes of fluid over specified periods of time. Commercially available, disposable micropipette tips are used with the CellSelector, and the tip is visible in the microscope's field of view so that cell and fluid manipulation can be readily observed. In addition to precisely manipulating minute volumes, the CellSelector is designed to accomplish the unambiguous selection and isolation of rare cells or particles. Applications include rare-cell isolation for cloning or genetic analysis, sorting of selected chromosome fragments, delivery of microdrops of stains or antibodies to obtain spatially limited chemical reactions, and collection and storage of cells or particles.

Reader Enquiry No. 101

Scalable purification

Beckman Instruments has designed ProScale to be a logical, systematic approach to **protein purification** to assist in the drug-development process. The system includes workstations, software and chemistries. It uses the scalable HyperD media, allowing customers to move protein-based drugs from research to production using the same media, for cost and time savings. According to the manufacturer, HyperD offers the flow characteristics of a rigid porous particle with the static binding capacity of a soft gel. The BioSys 500 and 2000 series workstations, are optimized to work with HyperD media, from BioSeptra. The new software on the BioSys 2000 contains several modules that are designed to speed method development through a logical pathway to reduce substantially the



The ProScale protein-purification system from Beckman.

trial-and-error nature of protein purification. A simulation option is designed to save time, effort and sample by allowing the researcher to explore separation and scale-up strategies without physically running the experiments. The software also builds a database on the protein, which can be used at subsequent stages through scale-up.

Reader Enquiry No. 102

Pharmacia Biotech has developed ÄktaExplorer, a new, **single-unit, high-performance liquid chromatography system**, developed to optimize purification methods for proteins, peptides, nucleic acids and oligonucleotides. It uses pre-set running parameters for about 80 pre-packed columns and prepares buffers automatically from stock solutions. The system is controlled and automated by Unicorn software. Its main instrument components are stacked on the left-hand side of the instrument, and the liquid-handling module mounted in a separate, enclosed section on the right. The entire system is

mounted on a swivel platform so that both sides can be easily swung to face the operator. ÄktaExplorer is designed to support all method development strategies and chromatographic techniques, such as ion exchange, hydrophobic interaction, reverse-phase, affinity and gel filtration. Built-in methods for a wide range of columns enable fast media screening, method scouting and scaling experiments. The system comes with pre-programmed protocols,

designed to ensure that users who have no time to develop their own purification strategies can achieve reliable results with minimum start-up time. Unicorn software is designed to control the entire process, regardless of location or scale of operation — from the laboratory bench to full production scale. BufferPrep, the automatic buffer preparation capability eliminates the time-consuming aspects of chromatography, such as buffer preparation and titration.

Reader Enquiry No. 103

Merck now offers the L-7650 **fraction collector** for preparative biochromatography. This unit was designed to be highly versatile. It can be used with numerous fractionation vessels, for example, from the smallest of fractions in Eppendorf tubes to litre-sized fractions in larger vessels. Fractionation can be completely automated, as the L-7650 can communicate directly with the chromatography system in use. The system also features a programming dialogue, a keyboard, liquid-crystal display and a small footprint.

Reader Enquiry No. 104

Waters has introduced Alliance **high performance liquid chromatography systems**, which incorporate integrated solvent and sample management technology. These new HPLC systems feature a solvent and sample management system, the 2690 separations module, which includes minimal system volume for more efficient separations a new solvent delivery technology that brings a high level of flow precision, simplified maintenance procedures for improved uptime, and a 25 per cent increase in sample capacity to enhance laboratory productivity. The separations module comes



Äkta for optimized purification methods.

independently with digitally controlled piston drives, for accurate and pulse-free isocratic- or gradient-solvent delivery. These systems compensate for changes in eluent viscosity and automatically purge any gaseous mobile phase. No tools are required to access high-pressure seals and pistons, and operators can change the seals without breaking fluid connections. The 2690 separations



The Alliance 2690 separations module features five, 24-position carousels.

module features five, 24-position carousels, providing high-capacity sample management with the flexibility for multiple-method or multiple-operator usage. While analysing one carousel of samples, other samples can be prepared and inserted into carousels, and then be loaded into the system without interrupting ongoing analyses. These systems feature a floppy disk drive for simple, error-free methods transfer. A real-time running log provides a permanent record of performance suitable for use in Good Laboratory Practice files or for monitoring preventive maintenance requirements. **Reader Enquiry No. 105**

Diagnostic division

Chiron offers its Quantiplex HIV RNA quantitative HIV bDNA probe assay. It utilizes the branched-DNA (bDNA) technology, as it is called, to quantify directly the amount of HIV in the plasma of people infected with the

ADVERTISEMENT

**Custom
MAb
Production**

- Hybridoma Development
- Ascites Production
- In-Vitro Production
- Purification Services
- Labeling • Fragmentation

(518) 537-8000



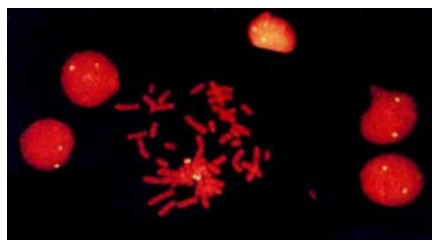
READER ENQUIRY NO 5



Peptide-N-glycosidase F (PNGase F), isolated from *Flavobacterium meningosepticum*, is now available from Oxford GlycoSystems as a recombinant product overexpressed in *Escherichia coli*. This product is available in a high-purity form and in bulk. Recombinant PNGase F should prove to be a suitable choice for controlled recovery of intact de-N-glycosylated peptides and proteins or selective recovery of intact N-linked oligosaccharides from glycoproteins. PNGase F is useful for determining the biological significance and functional role of N-glycosylation. Recombinant PNGase F is extensively purified to remove protease and contaminating glycosidase activities. The enzyme preparation is then screened for contaminating activities against synthetic chromogenic substrates and a range of naturally occurring oligosaccharides. PNGase F is supplied with a batch-specific specification sheet, protocol and glycerol-free incubation buffer. **Reader Enquiry No. 120**

AIDS virus. Measuring HIV levels with bDNA is designed to provide physicians with a prognostic alternative to CD4⁺ T-cell counts in determining disease progression. According to the manufacturer, viral load measurements to date demonstrate that direct quantification of HIV RNA by bDNA predicts HIV disease progression earlier and more accurately than CD4⁺ T-cell counts. Viral load is said to be a better predictor than CD4⁺ T-cell counts of how quickly people with HIV infection will progress to AIDS. **Reader Enquiry No. 106**

Oncor has released the MLL DNA probe for acute leukaemia research. The MLL gene is localized to chromosome IIq23 and is involved in translocations with more than twenty different genes. Rearrangements of this gene occur in



MLL DNA probe for leukaemia research.

5–10 per cent of all adult and paediatric acute leukaemias. Use of this probe in conjunction with the MLL Southern blot probe is said to allow detection of all the breaks that occur within the MLL gene. The probe is digoxigenin-labelled and should prove useful in bone marrow and peripheral blood studies. **Reader Enquiry No. 107**

Incstar has introduced the Pro-Trac II FK506 ELISA, its latest development in immuno-suppressive drug detection. This investigational kit is designed for the

quantitative determination of FK506 in human EDTA whole-blood samples. When defined as the apparent concentration at two standard deviations from the absorbance of the zero standard, its mean sensitivity is $0.18 \pm 0.03 \text{ ng ml}^{-1}$. A new addition to the kit is an enzyme digestion reagent extraction that is said to save time and eliminate the need for special equipment, as with methanol extraction methods. This competitive enzyme immunoassay employs a monoclonal antibody to FK506, runs on a microtitre plates, and includes standards and controls. Samples are extracted with an enzyme digestion reagent, then added to the wells of the microtitre plate, followed by addition of the anti-FK506 monoclonal antibody. After a 30-min incubation at room temperature, the FK506-horseradish peroxidase conjugate is added and incubated an additional 60 min. The wells are then washed and chromogen is added for a 15-min incubation. This reaction is stopped by addition of acid and the absorbance in each well is read at 450 nm and 630 nm. Concentrations are determined from a standard curve. **Reader Enquiry No. 108**

An ELISA kit designed to measure rat TNF- α , in serum, plasma or cell-culture fluids is now available from Genzyme. This kit provides a mass-calibrated, rat TNF- α , recombinant-protein standard, enough reagents for up to six standard curves and offers stability at 4 °C for the entire shelf-life of the kit. The stated sensitivity is 10 pg ml^{-1} , with results achievable in under four hours. In addition to the traditional single-plate format, this ELISA kit is also offered in bulk as 3-, 4-, 5- and 10-plate systems. This product adds to the company's range of rat recombinant proteins and antibodies for use with rat models. **Reader Enquiry No. 109**

Becton Dickinson Immunocytometry Systems (BDIS) has announced a new **system for measuring low, absolute CD4 counts. With the FACScout system, physicians can now have accurate absolute CD4 counts down to one cell per microlitre.** The system also measures absolute CD8 and CD3 counts down to one cell. Precise CD4 T-lymphocyte levels are a critical indicator in determining patient treatments for HIV, in monitoring therapy responses and in tracking disease progression. The system has been equipped with an RS232 port, allowing the instrument to be connected directly to IBM-compatible host computers. This allows patient results to be electronically transmitted to a laboratory information system. The FACScout system uses a three-step method for CD4, CD3 and CD8 counts, and is self-contained, elimi-



FACScout for CD4 measurement.

nating the need for additional instrumentation and simplifying the sample preparation process. It uses whole blood so that blood cells do not need to be lysed, reducing operator variability. Once a blood sample is analysed using this system, a summary printout of the absolute numbers of CD4, CD8 and CD3 T lymphocytes and a helper/suppressor ratio is printed from the built-in printer.

Reader Enquiry No. 110

Kamiya Biomedical has introduced an **anti-human LRP monoclonal antibody specific to the p110 protein** that can be used for the detection of LRP-associated, non-PgP multiple drug resistance (MDR). The LRP protein defines a cancer multiple drug resistance phenotype that is not mediated by P-glycoprotein (for example, non-PgP MDR). It is upregulated and strongly overexpressed in various human non-PgP MDR tumour-cell lines. The anti-human LRP monoclonal can be used for staining paraffin-embedded and frozen tissue sections. It can also be used for flow cytometry applications.

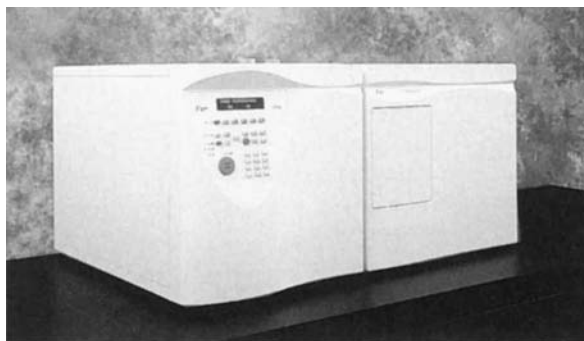
Reader Enquiry No. 111

Instrumental interest

Thermo-Umicam **capillary electrophoresis (CE) systems** are designed to be flexible, to be interfaced directly with a mass spectrometer (without modification), and to use a total capillary length as short as 35 cm. High-resolution separations by capillary electrophoresis can be a useful technique for the assessment of drugs in clinical research studies. However, the complexity of biological matrices can give rise to data uncertainties because of loss of peak resolution, migration time shifts, and comigration of analyte and matrix components. Therefore, confirmation by an independent test can be necessary — linking capillary electrophoresis with electrospray mass spectrometry offers this independent identification and enhanced detection. The high-voltage power supply of this system is compatible with a floating ground and, therefore, no electrical connection between the capillary electrophoresis and mass spectrometer is required. Dynamic compression injection is designed to ensure peak area RSDs of typically less than one per cent. The modular design makes adding on-line ultraviolet detection easy, and because the detector can be located a few centimetres from the electrospray inlet, UV and mass spectrometric electropherograms are virtually identical.

Reader Enquiry No. 112

Finnigan has announced the introduction of the Automass **benchtop GC/MS**. This gas chromatograph has been optimized to provide the best yield on high-boiling, high polarity or thermal-labile compounds. A software-controlled precision autosampler is said to ensure unattended high throughput. The mass spectrometer detector features a high-transmission quadrupole



The Automass GC/MS system from Finnigan is capable of compound identification of a 1,000K range.

analyser designed to allow rapid, reliable identification of compounds over a 1,000K range — even at trace levels. The instrument can operate in full spectra acquisition mode for maximum spectral information, as well as in single-ion monitoring mode for ultra-trace analysis and accurate quantitation. Direct insertion and desorption probes will allow compounds to

be inserted directly into the mass spectrometer in solid or diluted form, without prior separation. The system auto-tunes and auto-calibrates, and methods and operating parameters can be stored and reloaded at any time by using the Windows-based software.

Reader Enquiry No. 113

Neuroscience necessities

RBI now offers the p-MPPI, 5-HT_{1A} **serotonin receptor antagonist**. The high concentration of 5-HT_{1A} receptors in brain regions associated with mood and anxiety (for example, hippocampus, amygdala and septum) has prompted much interest in this serotonin (5-HT) receptor subtype. Research has been hampered by the lack of a selective, high-affinity 5-HT_{1A} receptor antagonist. Now there is p-MPPI, which has been shown to be a potent antagonist of central 5-HT_{1A} receptors. For example, in rat hippocampal homogenates 100 nM of p-MPPI completely blocks the 8-hydroxy-DPAT-induced inhibition of forskolin-stimulated adenylate cyclase activity. Furthermore, *in vitro* homogenate binding studies performed in rat hippocampal homogenates using ¹²⁵I-p-MPPI have revealed a significant increase in the number of binding sites in the presence of quanyl nucleotides. A number of cloned 5-HT serotonin receptors, supplied as frozen aliquots, are also available.

Reader Enquiry No. 114

Clonetics now offers NeuroPack-AS, a **complete cell system containing normal human astrocytes** (for example, non-transformed), optimized growth medium and subculture reagents. Astrocytes play a diverse and vital role in the development and normal functioning

ADVERTISEMENT

LIPOSOMES

REPRODUCIBLE — HOMOGENEOUS —
EXTREMELY RAPID

Our new LIPOSOMAT guarantees rapid (5ml within 60 minutes) preparation of uniformly sized unilamellar liposomes. Liposome size is selectable between 25 and ca. 600 nm diameter. Sample volume is normally 5-10ml or up to 200ml if used in combination with an additional instrument. Lipid concentration can be up to 300 mg/ml.

DIABORM

P.O. Box 650126, D-81215 Munich
For USA call 1-800 Liposome

READER ENQUIRY NO 12

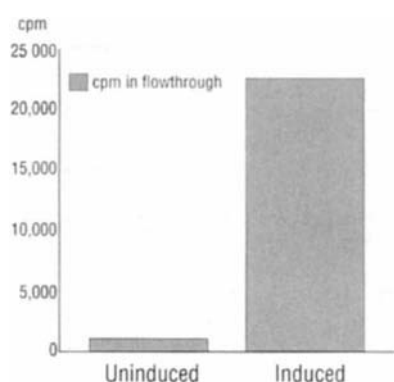
of the central nervous system. Much of what is known about astrocyte function comes from work with cell cultures. Cell characterization includes a positive stain for glial fibrillary acidic protein (GFAP) and a full certificate of analysis is sent out with all cells. The cells are supplied cryopreserved — with optimized media (AGM) and all the reagents necessary for subculture. AGM is modified MCDB 131 (CCMD 190), designed for the optimal proliferation of normal human astrocytes. This system consists of cryopreserved normal human astrocytes (5×10^5 cells per amp), one 500-ml bottle of optimized astrocyte growth medium and subculture reagents.

Reader Enquiry No. 115

DuPont NEN has launched four new growth factors to join its range of **tritiated and iodinated growth factors**. The new iodinated ligands fall into two groups: cytokines and nerve growth factors. They feature full biological activity, high specific activity and extended stability. The two new cytokines are ^{125}I -labelled derivatives of human recombinant IL-5 and human IL-10 and may be used in receptor binding and crosslinking studies. Both are available in 5 μCi and 25 μCi lyophilized packs and are said to be stable for at least four weeks. Possible applications include the elucidation of the role of individual cytokines in the regulation of cell-to-cell interactions in the initiation and maintenance of the inflammatory immune response in many diseases, including asthma, autoimmune diseases, such as rheumatism and arthritis, and viral diseases, such as AIDS. The two nerve growth factors are BDNF (brain-derived neurotrophic factor) and murine NGF, which may be used in studies of neuronal differentiation, survival and regeneration.

Reader Enquiry No. 117

Stratagene now offers a **nitric oxide synthase assay kit** to help investigate the biological functions of nitric oxide. The new NOSdetect assay kit is designed to



Stratagene's NOSdetect assay kit.

provide a rapid, sensitive means for testing the activity of nitric oxide synthases. The kit is based on monitoring the conversion of [^3H]arginine to [^3H]citrulline during the enzymatic formation of nitric oxide. This should prove useful for NOS activity in crude and purified enzyme preparations. The kit uses radioactive substrates [^3H]arginine or [^{14}C]arginine, making it sensitive in the picomole range. The kit's direct conversion of arginine to citrulline is specific for the NOS pathway, and as citrulline is easily separated from unreacted arginine, hundreds of assays can be performed in less than an hour, according to the manufacturer. Applications for this kit include drug screening, studies of neurotransmitters, vascular regulators and modulators of inflammatory processes.

Reader Enquiry No. 116

Neuroscience necessities

Heraeus Instruments has developed the BB 6220 M, a fully **automated, robotic, gas incubator**. The robot takes over routine microtitre plate testing in cell research and pharmaceuticals. The computer-controlled robot loads the carousel-shaped, networked magazine into the CO_2 incubator. The magazine can hold up to 135 microtitre plates (MTPs). In addition, the robot carries out pipetting, incubating, washing, separating and measuring. Each

microtitre plate is selected by the control system through the built-in computer interface, and the robot accesses it with a tolerance level of ± 0.3 mm. A precise, fast-reacting electric motor drives the MTP carousel directly. The automated incubator door helps to maintain a constant temperature by opening only 140 mm. This is enough space to provide access for most types of laboratory robots. The door also incorporates a heating system ensuring that the atmosphere inside the incubator recovers quickly. When an MTP is removed from the incubator, the drop in temperature that results from the door being opened is stated to be only 0.2 $^\circ\text{C}$. Moreover, the temperature is said to return to normal in about one minute.

Reader Enquiry No. 118

The Concentrator 5301 developed by Eppendorf is a flexible, modular system for gentle **concentration of DNA/RNA, nucleotides and proteins**, as well as for preparing gel electrophoresis. The user can choose between the basic concentrator model, which can be operated with an existing pump, or the complete system, which is equipped with a maintenance-free, chemical-resistant PTFE diaphragm pump. All components are either coated with PTFE or made of stainless steel for increased reliability and a long operating lifetime. The standard delivery package of the Concentrator 5301 includes a fixed-angle rotor for 48 $\times$ 1.5- or 2.0-ml micro-test tubes. The complete system also features an emission condenser, which cleans outgoing air up to 85 per cent. For larger numbers of samples, a 72-place rotor is available for 0.5 ml tubes or a 70-place plate rotor for 1.5- or 2.0-ml tubes. The capacity of the concentrator can be increased by stacking the fixed-angle rotors.

Reader Enquiry No. 119

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

ADVERTISEMENTS

OLIGONUCLEOTIDES

£1 per base

- stringent quality control
- express turnaround
- column purified: ready to use

By research labs for research labs

Molecular Biology Services

King's College School of Medicine and Dentistry
Rayne Institute, 123 Coldharbour Lane, London SE5 9NU
Tel: 0171-346 3126 Fax: 0171-733 3877

READER ENQUIRY NO 10

Gramsch Laboratories

- New: Somatostat. Rec. 2A/B Antisera
- G-Protein Antisera - α , β , γ -subunit
- G-Protein Peptide Fragments
- Opioid Receptor Antisera
- Monoclonal Opioid-Antibody 3E7
- YGGF... - specific; appl.: combinatorial peptide library tests
- Cyclic Nucleotide Antisera
- Antibody/Peptide Production Service

Phone 08138 1769 or 08131 5679-0
Fax 08138 1260 or 08131 5679-24
WWW:ourworld.compuserve.com/homepages/jgramsch/gl.htm

Gramsch GmbH

D-85247 SCHWABHAUSEN, Germany

NEWCASTLE UNIVERSITY
MOLECULAR BIOLOGY UNIT

PROTEIN SEQUENCING

Membrane-bound, solid or solution samples

£12 per cycle

5 cycles minimum - no set up charge

For further information contact Dr. Joe Gray
Phone: +44 191 222 8612
Fax: +44 191 222 8129
Email: JOE.GRAY@newcastle.ac.uk

READER ENQUIRY NO 12